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(57) Abstract: The invention encompasses recombinant polynucleotides, compositions and methods for interfering with antibiotic resistance genes, and/or replicons carrying such genes, in microorganisms in order to disable antibiotic resistance in the microorganisms, using a clustered regularly interspaced short palindromic repeat (CRISPR) array system.



Therapeutic

The invention relates to recombinant polynucleotides, compositions and methods for interfering with antibiotic resistance genes, and/or replicons carrying such genes, in
5 microorganisms in order to disable antibiotic resistance in the microorganisms.

Antibiotics, originally isolated from microorganisms such as *Streptomyces*, are a powerful way to treat infectious disease. However, very quickly bacteria acquired anti-microbial resistance (AMR) to antibiotics in response to selection pressure. One common route to
10 AMR has been the acquisition of resistance genes evolved in the original antibiotic-producing microorganisms, via horizontal transmission on plasmid vectors. Such plasmids have in some instances acquired multiple antibiotic resistance genes carried by transposable elements and integrons. Host-encoded mutations that modify the bacterial protein target or prevent entry of the antibiotic have also occurred.

15 Resistance to antibiotics by microorganisms such as bacterial pathogens is one of our most serious health threats. Infections from resistant bacteria, for example, are now not uncommon, and some pathogens have even become resistant to multiple types or classes of antibiotics. The loss of effective antibiotics undermines our ability to fight infectious diseases
20 and manage the infectious complications common in vulnerable patients, for example those undergoing chemotherapy for cancer, dialysis for renal failure, and surgery, especially organ transplantation, for which the ability to treat secondary infections is critical.

When first-line and second-line treatment options are limited by antibiotic resistance or are
25 unavailable, healthcare providers are forced to use alternative antibiotics that may be more toxic to the patient, more expensive and less effective. Even when alternative treatments exist, patients with resistant infections are often more likely to die, while survivors may have significantly longer hospital stays, delayed recuperation, and long-term disability.

30 Many achievements of modern medicine are put at risk by AMR. Without effective antibiotics for care and prevention of infections, the success of treatments such as organ transplantation, cancer chemotherapy and major surgery would be compromised.

The growth of global trade and travel allows antibiotic resistant microorganisms to be spread rapidly through humans, other animals, and food.

35 Resistance mechanisms fall into four classes:

- (1) enzymes that degrade antibiotics, including beta-lactamases that break the beta-lactam ring of the penicillin family of antibiotics;
- (2) enzymes that modify antibiotics include aminoglycoside phosphotransferases that phosphorylate aminoglycoside antibiotics such as kanamycin; chloramphenicol acetyl-
5 transferase (CAT) that acetylate chloramphenicol;
- (3) efflux pumps that actively export antibiotics from cytoplasm out of the cell, such as the tetracycline efflux pump that is expressed in the presence of tetracycline, plus other pumps, conferring multidrug resistance, that are capable of exporting a range of antibiotics; and
- (4) mutations that change the protein target of the antibiotic such that it is no longer
10 inactivated by it; for example, beta-lactams are bactericidal because they inhibit penicillin-binding proteins (PBPs) that are required for peptidoglycan biosynthesis and bacterial cell wall integrity and PBP mutants with reduced binding to beta-lactams will not be inhibited.

Several approaches are currently being used or developed to address the problem of
15 antibiotic resistance, including the following.

Firstly, new antibiotics, such as derivatives of existing drugs, have been developed. Fewer new antibiotic drugs have been developed, and many are more toxic so are used in the last resort. Microorganisms have acquired resistance to new antibiotics of both types.

20

Secondly, direct inhibition of resistance enzymes has been attempted. Examples include clavulanic acid, a beta-lactamase inhibitor which is used in combination with amoxycillin, a beta-lactam antibiotic (also called Augmentin). Other beta-lactamase antibiotic inhibitors include the carbapenems. But even here resistance has appeared. Blueberry Therapeutics
25 and Avacta are developing peptide affimers to target mechanisms of resistance.

Development of new inhibitors of antibiotic resistance enzymes requires a long pipeline of drug development. An alternative approach has been adopted by Dr Eric Brown at McMaster University, Canada, who is screening known drugs (already approved for use) with unrelated targets, for cryptic activity in inactivating antibody resistance mechanisms.

30

Thirdly, non-antibiotic bactericides have been used. For example, infection by bacteriophage was developed in the 1920's and although largely discontinued with the discovery of antibiotics, has been retained in certain countries. Current approaches use virulent, lytic bacteriophage that kill bacteria, including antibiotic resistant bacteria, but this opens the way
35 for selection of bacterial variants that are resistant to bacteriophage infection. To obviate this, preparations containing a mixture of different strains of bacteriophage are being used.

Another disadvantage of the use of such lytic bacteriophage in patients suffering from sepsis

is that cell lysis and death by lytic bacteriophage can release endotoxins from the cell into the blood and can cause endotoxin shock.

Further compositions and methods for combating antibiotic resistant microorganisms are
5 required.

According to a first aspect of the present invention, there is provided a recombinant polynucleotide comprising a clustered regularly interspaced short palindromic repeat (CRISPR) array nucleic acid sequence having or transcribing an RNA guide molecule with a
10 spacer sequence sufficiently complementary to a target sequence of an antibiotic resistance gene in a microorganism for the antibiotic resistance gene to be inactivated in the presence of a CRISPR associated (Cas) DNA-binding polypeptide or a functional equivalent or a modified version thereof, thereby sensitising the microorganism to the antibiotic.

15 In another aspect of the invention, there is provided a delivery vehicle for introducing a polynucleotide into a microorganism, such as an antibiotic-resistant microorganism, in which the delivery vehicle comprises a recombinant polynucleotide for inactivation of DNA carrying a gene encoding an antibiotic resistance enzyme which confers antibiotic resistance to the microorganism, and in which the recombinant polynucleotide comprises a clustered regularly
20 interspaced short palindromic repeat (CRISPR) array nucleic acid sequence having or transcribing an RNA guide molecule with a spacer sequence sufficiently complementary to a target DNA sequence of the antibiotic resistance gene for the antibiotic resistance gene to be targeted and inactivated in the presence of a CRISPR associated (Cas) DNA-binding polypeptide or a functional equivalent or a modified version thereof, thereby sensitising the
25 microorganism to the antibiotic, wherein the delivery vehicle is a non-virulent or a lysogenic bacteriophage.

One general aim of the present invention is inactivation of DNA carrying a gene encoding an antibiotic resistance enzyme using a CRISPR/Cas system. An advantage of the invention is
30 that one or more existing antibiotics can be used to treat infectious disease, as microorganisms become re-sensitised to the antibiotics or are prevented from acquiring antibiotic resistance.

The target sequence of an antibiotic resistance gene may be a sequence flanking the gene
35 itself which, if disrupted, inactivates the antibiotic resistance gene. For example, if the antibiotic resistance gene is located on a plasmid, the invention may encompass a target sequence in the plasmid.

In contrast to prior art approaches of inactivating antibiotic resistance enzymes, the present invention will not require new drug development and the concomitant regulatory approval required for each new drug. Rather, the invention provides a tool which can be applied to
5 target and inactivate relevant antibiotic resistance genes directly rather than the gene products. For example, a gene encoding an antibiotic resistance enzyme, or a gene encoding a protein regulating the uptake and export of an antibiotic by altering the membrane permeability and efflux pump expression, respectively, can be targeted.

10 The CRISPR/Cas system is an RNA-mediated genome defense pathway that is part of a natural bacterial and archaeal immune system against nucleic acid invaders, analogous to the eukaryotic RNAi pathway (see for example Grissa et al., 2007, BMC Informatics 8: 172; Horvath & Barrangou, 2010, Science, 327: 167–170; Gasiunas et al., 2012, Proc. Natl Acad. Sci. USA 109: E2579; Marraffini & Sontheimer, 2008, Science, 322: 1843-1845; Garneau
15 et.al.,2010, Nature 468: 67). Natural CRISPR systems contain a combination of Cas genes as well as non-coding RNA elements capable of programming the specificity of the CRISPR-mediated nucleic acid cleavage. Three types (I-III) of CRISPR systems have been identified thus far in a wide range of bacterial and archaeal hosts. Each CRISPR locus is composed of a series of short DNA direct repeats separated by non-repetitive spacer sequences. The
20 spacer sequences, in nature, typically originate from foreign genetic elements such as bacteriophage and plasmids. As used herein, the series of repeats plus non-repetitive spacer sequences is known as a CRISPR array. The CRISPR array is transcribed and hybridised with repeat complementary tracrRNA followed by cleavage within the direct repeats and processed into short mature dual tracrRNA:crRNAs containing individual spacer sequences,
25 which direct Cas nucleases to a target site (also known as a “protospacer”). For example, the Type II CRISPR/Cas9 system, a well-studied example, carries out a targeted DNA double-strand break (“DSB”) in four steps. Firstly, two RNAs, the pre-crRNA array and tracrRNA, are transcribed from the CRISPR locus. Secondly, tracrRNA hybridises to the repeat regions of the pre-crRNA and mediates the processing of pre-crRNA into mature crRNAs (also referred
30 to herein as “RNA guide molecules gRNA” containing individual or monomer spacer sequences. Thirdly, the mature crRNA:tracrRNA complex directs Cas9 protein in the form of a ribonucleoprotein to the target DNA via base-pairing between the spacer on the crRNA and the target site on the target DNA. Finally, Cas9 mediates cleavage of target DNA and creates a DSB.

35

In the present invention, as elaborated herein, modified CRISPR constructs are used to target antibiotic resistance genes. The recombinant polynucleotide of the invention using

such a construct is also referred to herein as an “assassin construct” which is used to effect inactivation of such genes.

- The main focus of using CRISPR technology to date has been for use as a DNA editing tool
- 5 for reverse genetics, primarily in eukaryotes. However, WO2007/025097 describes the use of CRISPR technology for modulating resistance in a cell against an invading target nucleic acid or a transcription product thereof, especially against invading bacteriophages. Methods for downregulating prokaryotic gene expression using CRISPR technology to target mRNA transcribed by the genes have been suggested for example in WO2010/075424.
- 10 WO2012/164565 describes a CRISPR system from *Lactococcus* and use of the system for modulating resistance of a cell against an invading target nucleic acid or a transcription product thereof. The present invention, by contrast, concerns *inter alia* inactivation in an antibiotic-resistant microorganism of genes involved in conferring the antibiotic resistance.
- 15 According to the invention, the RNA guide molecule may mediate binding of the Cas DNA-binding polypeptide or its functional equivalent or its modified version to the antibiotic resistance gene. This mirrors the natural system described above.

- The Cas DNA-binding polypeptide or its functional equivalent or its modified version of the
- 20 invention may also be capable of binding to RNA or other nucleic acid molecules. In other words, the requirement for the Cas DNA-binding polypeptide or its functional equivalent or its modified version to be capable of binding DNA does in some aspects of the invention does not exclude the polypeptide or its functional equivalent or its modified version being capable of binding RNA or other nucleic acid molecules. In these aspects, the Cas DNA-
- 25 binding polypeptide or its functional equivalent or its modified version may be referred to as a Cas nucleic acid-binding polypeptide or its functional equivalent or its modified version.

- For certain applications, the microorganism may have a natural endogenous, or introduced engineered, Cas DNA-binding polypeptide or functional equivalent or modified version. This
- 30 means that the recombinant polynucleotide of the invention is not required to encode the Cas DNA-binding polypeptide or functional equivalent or modified version. Alternatively, the recombinant polynucleotide of the invention may further comprise a nucleic acid sequence which encodes the Cas DNA-binding polypeptide or its functional equivalent or modified version. In another aspect, the recombinant polynucleotide of the invention does not encode
- 35 the Cas DNA-binding polypeptide or its functional equivalent or modified version but may be used in conjunction with a separate polynucleotide which does. Other means for introducing

the Cas DNA-binding polypeptide or its functional equivalent or its modified version into the microorganism may be used.

An exemplar Cas DNA-binding polypeptide according to the invention is Cas9 or a functional
5 equivalent thereof or a modified version thereof.

In the recombinant polynucleotide according to various aspects of the invention, the CRISPR array nucleic acid sequence may have or transcribe additional RNA guide molecules each comprising a spacer sequence sufficiently complementary to a target sequence of the
10 antibiotic resistance gene or one or more additional antibiotic resistance genes. The or each RNA guide molecule may be transcribed from its own promoter sequence. Alternatively, a set of a number of RNA guide molecules may be transcribed from one promoter sequence and optionally in combination with one or more other such sets. For example, a set of four RNA guide molecules may be transcribed from one promoter sequence, for example in
15 combination with one or more other such sets of guide molecules.

Having multiple RNA guide molecules allows different antibiotic resistance (or other types of) genes in a microorganism to be targeted and inactivated simultaneously.

20 The recombinant polynucleotide according to various aspects of the invention may additionally or alternatively be designed to include an RNA guide molecule (such as a further RNA guide molecule) targeting a gene involved in pathogenicity or other aspects of microbial metabolism. For example, certain pathogens form biofilms that make it difficult for antibiotics to gain access to them. One or more genes involved in bacterial metabolism for biofilm
25 production may be targeted.

Spacer sequence distal from a promoter are typically less efficiently transcribed. Ideally, multiple RNA guide molecules to different targets should be more or less equally represented. Thus, one promoter transcribing each RNA guide molecule may be used
30 (instead of relying on a long polycistronic RNA guide molecule [or precursor crRNA] transcription).

For example, there are many resistance genes encoding beta-lactamases (*bla* genes) giving resistance to a large range of different beta-lactam antibiotics. DNA constructs expressing
35 multiple RNA guide molecules, which may each be individually transcribed from their own such promoters, may be used to target a number of different *bla* genes.

Thus in aspects of the invention, the CRISPR array nucleic acid sequence may have or transcribe one or more RNA guide molecules each comprising a spacer sequence sufficiently complementary to a target sequence of one or more beta-lactamase genes.

- 5 For example, the one or more RNA guide molecules may target one or more or all of the genes selected from the group consisting of : NDM, VIM, IMP, KPC, OXA, TEM, SHV, CTX, OKP, LEN, GES, MIR, ACT, ACC, CMY, LAT, and FOX.

- In particular, the one or more RNA guide molecules may comprise a spacer sequence
- 10 sufficiently complementary to target sequences of the beta lactam family of antibiotic resistance genes, including one or more or all of the following: a first spacer sequence sufficiently complementary to target sequences for NDM-1, -2, -10; a second spacer sufficiently complementary to target sequences for VIM-1, -2, -4, -12, -19, -26, -27- 33, 34; a
- 15 third spacer sufficiently complementary to target sequences for IMP-32, -38, -48; a fourth spacer sufficiently complementary to target sequences for KPC- 1, -2, -3, -4, -6, -7, -8, -11, -12, -14, -15, -16, -17; a fifth spacer sufficiently complementary to target sequences for OXA-48; a sixth spacer sufficiently complementary to target sequences for TEM-1,-1B, -3, -139, -162, -183, -192, -197, -198, -209, a seventh spacer sufficiently complementary to target sequences for SHV and its variants; and an eighth spacer sufficiently complementary
- 20 to target sequences for CTX and its variants.

- The antibiotic resistance gene to be inactivated may be located on a chromosome, or on an extrachromosomal replicating DNA molecule known as a replicon and including plasmids and bacteriophage.
- 25

- The CRISPR/Cas system used according to an aspect of the invention generates a DSB in the target sequence. Where the target sequence is located on a chromosome or a replicon such as a bacterial chromosome or plasmid, then a DSB can lead to degradation and hence loss of the chromosome or replicon suffering such a DSB. If the target sequence is
- 30 located on a bacterial chromosome then the cell may die directly as a consequence of the DSB. Additionally, some plasmids (including natural plasmids) carry killing functions that only become toxic if the cell loses the plasmid, which is a natural mechanism to ensure faithful inheritance of plasmids in dividing cells. If a plasmid carrying the target sequence of the antibiotic resistance gene also carries such a killing function, and the plasmid is lost as
- 35 a result of the DSB generated, the cell may die (see Sengupta & Austin, 2011, Infect.

Immun. 79: 2502-2509).

In the event that cell death caused by such DSB increases selection pressure for resistance against the recombinant polynucleotide according to various aspects of the present
5 invention, this may be mitigated by, for example, employing a modified Cas DNA-binding polypeptide which seals the target site after generating a deletion to inactivate the target sequence of the antibiotic resistance gene, rather than generate a DSB.

Thus the Cas DNA-binding polypeptide according to various aspects of the invention may in
10 certain aspects be substituted by a modified Cas DNA-binding polypeptide comprising a recombinase catalytic domain, wherein the modified Cas DNA-binding polypeptide does not generate DSBs but creates a deletion and reseals a site in the target sequence.

The modified Cas DNA-binding polypeptide may for example be a modified Cas9 protein
15 comprising a recombinase catalytic domain.

The recombinant polynucleotide according to various aspects of the invention may further comprise a nucleotide sequence which encodes a gene conferring a selective advantage to the microorganism, for example thereby increasing the efficiency of delivery of the
20 CRISPR/Cas system to the target microorganism. For example, the gene may confer a growth advantage over non-infected siblings, or genes encoding a bacteriocin – these are protein toxins produced by bacteria to kill or inhibit growth of other bacteria - and corresponding immunity polypeptide may be used.

25 The selective advantage to the microorganism may include or be one which prevents or diminishes the effect of loss of a replicon due to a DSB caused by Cas DNA-binding polypeptide. For example, the nucleotide sequence which encodes a gene conferring a selective advantage to the microorganism may encode an antitoxin that neutralises the effect of a toxin or killer function carried by a replicon on which the target sequence is
30 located. Also, the nucleotide sequence which encodes a gene conferring a selective advantage to the microorganism may encode one or more proteins that are encoded by a replicon subject to degradation due to a DSB caused by Cas DNA-binding polypeptide.

In another aspect of the invention, there is provided a delivery vehicle for introducing a
35 polynucleotide into a microorganism, in which the delivery vehicle comprises the recombinant polynucleotide as defined herein.

The delivery vehicle according to various some aspects of the invention may be a bacteriophage. Alternatively, the delivery vehicle may be a plasmid such as a conjugative plasmid or other plasmid replicon, a nucleotide vector, linear double-stranded DNA (dsDNA), an RNA phage or an RNA molecule.

5

The delivery vehicle may be a non-virulent bacteriophage, such as bacteriophage M13, a filamentous phage that infects *Escherichia coli* cells, replicates and is secreted from the bacterial host cell without killing the bacterial host, which continues to grow and divide more slowly. Another suitable filamentous phage is NgoPhi6 isolated from *Neisseria gonorrhoeae* that is capable of infecting a variety of Gram negative bacteria without killing the host.

10

Alternatively, lysogenic phage may be used that do not always kills host cells following infection, but instead infect and become dormant prophage.

15 This invention may thus use a novel system to inactivate antibiotic resistance genes in bacteria primarily using bacteriophage that do not kill bacteria, and/or conjugative plasmids and/or direct DNA transformation, as the delivery mechanisms for the recombinant polynucleotide of the invention.

20 In a further aspect of the invention, there is provided a composition comprising the recombinant polynucleotide as defined herein, or the delivery vehicle as defined herein.

The composition may be a pharmaceutical composition, a non-pathogenic microorganism such as a commensal bacterium for example in a probiotic formulation, or a dietary

25 supplement.

As used herein, a "pharmaceutical composition" refers to a preparation of one or more of the active agents (such the recombinant polynucleotide or the delivery vehicle as described herein) with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of the active agent to an organism.

30

The composition may be formulated for topical, enteral or parenteral administration.

35 Compositions of the present invention may, if desired, be presented in a pack, dispenser device or kit, each of which may contain one or more unit dosage forms containing the active

agent(s). The pack, dispenser device or kit may be accompanied by instructions for administration.

Also provided according to the invention is the composition as defined herein for use as a
5 medicament.

In another aspect there is provided according to the invention a composition as defined herein for use in the treatment or prevention of an infection caused by an antibiotic-resistant microorganism comprising an antibiotic resistance gene targeted by the RNA guide molecule
10 of the recombinant polynucleotide.

The invention further provides a method of treating or preventing an infection in a subject caused by an antibiotic-resistant microorganism comprising an antibiotic resistance gene, in which the method comprises the step of introducing into the microorganism a therapeutically
15 effective amount of the composition as defined herein where the RNA guide molecule targets the antibiotic resistance gene, thereby inactivating the antibiotic resistance gene and sensitising the microorganism to the antibiotic.

The composition may be administered topically or orally. Alternatively the composition may
20 be administered by intravenous, parenteral, ophthalmic or pulmonary administration.

Compositions of the present invention for administration topically can be in a form suitable for topical use such as, for example, an aerosol, cream, ointment, lotion or dusting powder.

25 Compositions provided herein may be formulated for administration by inhalation. For example, the compositions may be in a form as an aerosol, a mist or a powder. Thus compositions described herein may be delivered in the form of an aerosol spray presentation from pressurised packs or a nebuliser, with the use of a suitable propellant such as for example dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon
30 dioxide or other suitable gas. Where using a pressurised aerosol, a dosage unit may be determined by providing a valve to deliver a metered amount.

In the method, the subject may be a plant, a fish, a bird, a reptile or a mammal (such as a human).
35

According to the method, the delivery vehicle may be transferred from the microorganism directly into another microorganism (such as antibiotic-resistant microorganism) by plasmid conjugation or bacteriophage infection.

- 5 The method may further comprise a step of simultaneously or subsequently administering to the subject an antibiotic to which a microorganism has become sensitised.

- A further aspect of the invention provides a method of inactivating antibiotic resistance in a microorganism, the method comprising introducing into the microorganism of the
10 recombinant polynucleotide as defined herein, or the delivery vehicle as defined herein. The method may be an *in vivo* method applied to a subject, or an *in vitro* method.

- Also provided according to the invention is a host cell comprising the recombinant polynucleotide defined here. The host cell may, for example, be a commensal bacterium.
15

- Microorganisms targeted by various aspects of the invention may be on a body surface, localised (for example, contained within an organ, at a site of a surgical wound or other wound, within an abscess), or may be systemic. Included is the treatment of bacterial infections that are amenable to therapy by topical application for example using
20 bacteriophage of the invention.

- The present invention also encompasses coating of surfaces other than body surfaces with the recombinant polynucleotide, delivery vehicle or composition of the present invention, for example wound dressings or medical device surfaces.
25

Further aspects, features and non-limiting embodiments of the present invention will now be described below with reference to the following drawings:

- Figure 1. CRISPR/Cas9-mediated bacterial immunisation against antibiotic resistant genes on a plasmid:** I, CRISPR/Cas locus, where boxes denote different spacer sequences targeting different antibiotic resistance genes; II, gRNA-Cas9 where boxes denote different gRNAs targeting each antibiotic resistance gene; III, plasmid harbouring antibiotic resistance gene; IV, target recognition and positioning of Cas9; V, cleaved plasmid, VI: bacteriophage. **Ap^R** = ampicillin resistant, **Cm^R** = chloramphenicol resistant, **Km^R** = kanamycin resistant. **Ap^S** = ampicillin sensitive, **Cm^S** = chloramphenicol sensitive, **Km^S** = kanamycin sensitive. **Bla**= beta-lactamase, **CAT** = chloramphenicol acetyl transferase, **APH** = aminoglycoside phosphotransferase. **1. Injection** - CRISPR/Cas9 is injected into the bacterial cell along with phage DNA by bacteria-specific phage infection. **2. Lysogenisation** - Phage DNA is lysogenised and integrates into the bacterial host chromosome (**B. chr.**). **3. crRNA biogenesis and assembly of Cas9** - Pre-crRNA is transcribed and hybridised with tracrRNA and processed to make mature crRNA:tracrRNA (an RNA guide molecule, or "gRNA"), which is assembled with Cas9. **4. Recognition** - These gRNA-Cas9 complexes recognise target DNA on the plasmid. **5. Cleavage** - The gRNA-Cas9 complexes cleave DNA at the sites recognised by crRNAs. **6. Inactivation** - This leads to inactivation of the production of antibiotic resistant enzymes; **7. Sensitive** - Thus, the bacterial cell becomes sensitive to various antibiotics.

- Figure 2. Preventing non-pathogenic bacteria and asymptomatic pathogens from a future encounter with antibiotic resistance genes:** I, CRISPR/Cas locus, exemplified here as present in the bacterial chromosome (**B. Chr.**) and where boxes denote different spacer sequences targeting different antibiotic resistance genes; II, gRNA-Cas9, where boxes denote different gRNAs targeting each antibiotic resistance gene; III, relaxase –an enzyme that makes a strand-specific, sequence-specific nick in double-stranded DNA to initiate conjugal transfer of plasmid DNA; IV, bacteriophage, V, plasmid; VI, double-stranded DNA; VII, single-stranded DNA. Three entry pathways to encounter future antibiotic resistance genes are shown. **1. Transformation**- Entry of naked DNA carrying antibiotic resistance gene into the bacterial cell, **2. Bacteriophage-mediated transduction**- Phage carrying antibiotic resistance gene infects a bacterial cell. **3 Conjugation**- Plasmid carrying antibiotic resistance gene is conjugally transferred from a donor bacterial cell to a recipient bacterial cell. When these antibiotic resistance genes are introduced into the "immunised" cell; it is

pre-armed with a CRISPR/Cas locus encoding a gRNA-Cas9 that finds the specific target sequence on the antibiotic resistance gene and cleaves it to disrupt the gene.

5 **Figure 3. Re-sensitising symptomatic pathogens to antibiotics:** I, CRISPR/Cas locus in the bacterial chromosome (**B. Chr.**), where boxes denote different spacer sequences targeting each antibiotic resistance gene; II, gRNA-Cas9, where boxes denote different gRNAs targeting each antibiotic resistance gene; III, antibiotics; IV, protein synthesis is disrupted by cleaving corresponding gene; V, plasmid. This figure shows an asymptomatic
10 pathogen becoming symptomatic because of an opportunistic infection. The pathogen contains plasmid DNA that provides various antibiotic resistance agents such as **1. Efflux pumps**- capable of pumping antibiotics out of the bacterial cell, **2 Antibiotic degradation enzymes**-and **3. Antibiotic modification enzymes**. Each antibiotic resistant agent is disrupted (**X**) by gRNA-Cas9-mediated site-specific cleavage of corresponding genes.
15

Figure 4. Three possible CRISPR/Cas delivery routes: I, CRISPR/Cas locus where boxes denote different spacer sequences targeting each antibiotic resistance gene; II, relaxase—an enzyme that makes a strand-specific, sequence-specific nick in double-stranded DNA to
20 initiate conjugal transfer of plasmid DNA; III, bacteriophage; IV, plasmid; V, double-stranded DNA; VI, single-stranded DNA. This figure shows three possible delivery routes of CRISPR/Cas9 expression construct. **1. Transformation**- Entry of naked DNA carrying CRISPR/Cas9 into the bacterial cell via a DNA receptor on the cell surface, followed by integration (4) into the bacterial chromosome (**B. Chr.**) , **2. Bacteriophage-mediated**
25 **transduction**- Phage carrying CRISPR/Cas9 infects a bacterial cell, followed by circularisation (5) and then phage-mediated integration (4) into the bacterial chromosome (**B. Chr.**). **3 Conjugation**- Plasmid carrying CRISPR/Cas9 is conjugally transferred from a donor bacterial cell to a recipient bacterial cell, followed by circularisation (5) and plasmid replication. In all cases, boxes in the CRISPR/Cas9 locus denote different spacer sequences
30 targeting each antibiotic resistance gene.

Figure 5. Structure of bidirectional antibiotic resistant transmission model (see also Am J Epidemiol. 2013;178(4):508-520) and the effect of antibiotic resistance gene
35 **inactivation therapy:** **A**, Antibiotic Resistance is Dominant; **B**, Antibiotic Sensitivity is Dominant; **S**, Susceptible, **AS**, Antibiotic Sensitive; **AR**, Antibiotic Resistant; **T**, Transmission; **R**, Recovery, **C**, Conversion, **CCD**, CRISPR/Cas9 delivery. Antibiotic

resistance genes in an antibiotic resistant state and the encounter of antibiotic resistance genes in a sensitive state are disrupted for example by gRNA-mediated Cas9 cleavage. Antibiotic resistance gene inactivation therapy converts the bidirectional model (A) to an almost unidirectional conversion model (B) by increasing the conversion rate from antibiotic resistant to sensitive and decreasing the conversion rate from antibiotic sensitive to resistant. The area of each square represents the population density of each state. The width of the arrows is proportional to the magnitude of each parameter (transmission, recovery and conversion), i.e. transmission of antibiotic resistant bacteria is assumed to be higher than antibiotic sensitive bacteria in this figure. Recovery from the infection of pathogens sensitive to antibiotics is higher than for antibiotic resistant pathogens. In the presence of antibiotics, antibiotic-sensitive pathogens are converted to antibiotic resistant pathogens much more than the reverse conversion from resistant to sensitive pathogens because of the selection pressure. The treatment aim is to reverse these conversion parameters to drive the antibiotic resistant pathogen population to a sensitive state by introducing CRISPR/Cas9 into the bacteria.

Figure 6. Structure of superinfection antibiotic resistant transmission model and the effect of antibiotic resistance gene inactivation therapy: The bidirectional antibiotic resistance transmission model depicted in Figure 5 can additionally or alternatively be described by the following superinfection antibiotic resistance transmission model (see also Am J Epidemiol. 2013;178(4):508-520, as for Figure 5). When the antibiotic resistance genes are disrupted for example by gRNA-mediated Cas9 cleavage, this allows the population shift from the antibiotic resistant state to the antibiotic sensitive state via the superinfection state. Disruption of antibiotic resistance genes increases the conversion rate from the antibiotic resistant to the sensitive state. This figure gives a comparison of the relative population density before (Figure 6A) and after (Figure 6B) applying the CRISPR-Cas system. The population is composed of **S**, Susceptible; **lw**, sensitive; **lz**, resistant; and **lwz**, superinfection state. The area of each circle is proportional to the relative population density in each state. In this simulation, initial density in each state is assumed to be identical (0.25 each, represented by a thin-lined circle). Thick lined circles represent the population density at the equilibrium state. CRISPR-Cas system is clearly contributing to an increase of the population in the susceptible state, thus the recovery rate in the equilibrium state (i.e. S is expanding in B) and to reduce the population in the resistant state (i.e. lz is shrinking in B). The width of the arrows is proportional to the magnitude of each parameter (infection, recovery and state conversion), i.e. the infection of antibiotic resistant bacteria is assumed to be lower than that of antibiotic sensitive bacteria in this figure. Recovery from the infection of

the antibiotic sensitive pathogens is higher than that from the infection of the antibiotic resistant pathogens, because antibiotics are effective on the sensitive strains. In the presence of antibiotics, the antibiotic sensitive pathogens are converted to the antibiotic resistant pathogens much more than the reverse conversion because of the antibiotic

5 selection pressure. The aim of the treatment is to reverse these conversion parameters and to drive the antibiotic resistant pathogen population to the sensitive state.

Figure 7. Structure of CRISPR locus: This figure shows CRISPR locus containing six

10 spacers targeting six different regions. Each crRNA is transcribed monocistronically from the same promoters denoted P to control the transcription level identical for each target. Each crRNA transcript starts with a leader sequence L and terminates with a terminator sequence T. Transcripts of each pre-crRNA are shown as arrows and boxes containing different spacer sequences are indicated by unique shading.

15

Figure 8. Mapping short bacterial off-target sequence on the *bla* gene sequence. The figure shows the local alignment of bacterial short sequences (SEQ ID NOs: 3-5, 8-14, 17-23 and 26-33) mapped to the beta lactamase gene. Beta lactamase sequence (SEQ ID NOs: 1-

20 2, 6-7, 15-16 and 24-25) is shown in grey in the top of each panel, PAM (protospacer adjacent motif) sequence is shown in black. Base pairing region with crRNA is underlined, off-target seed sequence on the bacterial genome is italicised. Off-target seed sequence, including PAM sequence, is indicated by two vertical lines. Two numbers on the sequence of beta lactamase gene are the expected two base positions where the phosphodiester bond

25 is cleaved by Cas9 (see Example 1 below).

Figure 9. Predicted crRNA secondary structure. With reference to Figure 8 and Example 1, predicted secondary structures of the crRNA sequences using mFold are shown. The

30 sequences of CR05, CR30, CR70 and CR90 shown in Figure 9 correspond to SEQ ID NOs: 34-37, respectively.

Figure 10. Expected cleavage positions on beta-lactamase gene from pBR322 (SEQ ID

35 **NO: 38).** Two protospacer sequences, which are base-pairing with crRNA spacer sequences, are underlined (protospacer strand). Two anti-protospacer sequences, which are displaced when protospacer sequences are base pairing with crRNA spacer sequences,

are bold italicised (anti-protospacer strand). Associated PAM sequences are indicated either boxed small letters (on protospacer strand) or boxed capital letters (on anti-protospacer strand). Expected cleavage positions are indicated by asterisks. Leader sequence is underlined from 1st to 69th base and highlighted in grey.

5

Figure 11. Photograph of electrophoretically separated DNA products on a 0.8% agarose gel showing PCR amplicons generated in Example 2 from each of the three regions plus the EcoRV digested vector pACYC184. The marked lanes are as follows: 1* -

- 10 NEB (N3232S) 1 Kb molecular markers, 2 µg/lane; 2 - Fragment 1, tracrRNA-Cas9 region : 4758 bp; 3* - NEB (N3232S) 1 Kb molecular markers, 0.75 µg/lane; 4 - pACYC184 uncut; 5 - pACYC184 EcoRV cut : 4245 bp; 6 - pACYC184 EcoRV cut : 4245 bp; 7* - NEB (N3232S) 1 Kb molecular markers, 0.12 µg/lane; 8 - Fragment 2, leader and first direct repeat region : 276 bp; 9* - NEB (N3232S) 1 Kb molecular markers, 0.2 µg/lane; and 10 - Fragment 3,
- 15 Second direct repeat region : 452 bp. Note: * The mass of the each PCR amplicon is estimated by comparing the intensity of the appropriate marker band using NIH ImageJ 1.48v software(<http://imagej.nih.gov/ij/>).

- 20 **Figure 12. Plasmid map of pNB100 constructed in Example 2.** The plasmid map was drawn by SnapGene viewer ver. 2.4.3 free version (<http://www.snapgene.com>). Two direct repeats (DR) are shown as narrow white rectangular boxes adjacent to the 3' end of leader sequence.

25

Figure 13. Photographs show results of “Nemesis symbiotic activity” (NSA) according to an embodiment of the invention by bacterial cell mating (see Example 2). The left plate shows JA200 pNT3 x DH5α pNB100 in Ap100Cm35, while the right plate shows JA200 pNT3 x DH5α pNB102 in Ap100Cm35, both plated at 5×10^7 cells /ml.

30

- Figure 14. Photographs show results of NSA according to another embodiment of the invention by plasmid transformation (see Example 2).** Left: LB Cm35 plate. Colonies 1-40 are DH5α pBR322 transformed with pNB102; Colonies 45-60 are DH5α pBR322
- 35 transformed with pNB100. All colonies show resistance to Cm carried on plasmids pNB100 and pNB102; Right: LB Ap100 plate. Note that colonies 1-40 have lost ApR following transformation with pNB102 carrying the spacer region targeted against the beta-lactamase

gene carried on the plasmid pBR322 in strain NBec001, thereby demonstrating Nemesis symbiotic activity. pNB100 lacking this spacer region but carrying the Cas9 gene is unable to inactivate the beta-lactamase gene.

5

Figure 15. Shows a phagemid of Ngophi6. ORF11 and ORF7 of Ngophi6 are deleted from Ngophi6 genome. Coding sequences are represented by the arrows indicating the translation polarity of each ORF. The corresponding gene nomenclature of each Ngophi6 phage ORF to M13 are ORF1(gII), ORF2 (gV), ORF4 (gVIII), ORFV (gVIII), ORF8 (gVI), ORF9 (gI). M13 gene nomenclatures are in the parenthesis. The second T of the putative left integration site L = CTTATAT is changed to A to give L' = CATATAT to avoid integration. MCS = Multiple cloning site. The location of Ngophi6 and M13mp18 are indicated by the large two open arrows.

15

Figure 16. Shows a set of spacer sequences (SEQ ID NOs: 39-60) that encode 20 guide RNA molecules targeted against 117 different *bla* genes identified in the NCBI ARDB database for *Klebsiella pneumoniae* (see Example 5). Candidate spacer sequences were identified to disrupt all the *Klebsiella pneumoniae* beta lactamase genes found in the ARDB database. Beta lactamase gene sequences are collected from the ARDB database with the keyword *Klebsiella pneumoniae*. Redundant sequences were removed and unique sequences used for multiple sequence alignment using web program Clustal Omega. One canonical sequence was chosen from each cluster and the 20 nt spacer sequences predicted by the web program Jack Lin's CRISPR/Cas9 gRNA finder were collected. The spacer sequence is chosen to maximise the ratio of the proto-spacer sequence found in the sequences belonging to the same branch. Thus each of the example spacer sequences shown in the 4th column has the capability to disrupt the genes in the third column. Beta lactamase genes used in this analysis are: SHV-a = 1, 2, 2a, 5, 5a, 11, 12, 14, 26, 27, 28, 31, 33, 38, 43, 44, 48, 55, 56, 60, 61, 62, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 85, 89, 92, 98, 99, 101, 103, 106, 107, 108, 109, CTXM-b = 1, 3, 10, 12, 15, 22, 32, 54, 60, 62, 68, CTXM-c = 13, 14, 16, 18, 19, 24, 26, CTXM-d = 2, 35, 59, CTXM-e = 26, 63, TEM-f = 1, 1b, 3, ESBL, 139, KPC-g = 1, 2, 3, 4, OKP-h = A11, A12, A16, A17, B13, B-SB613, 6, LEN-i = 2, 17, 18, 19, 20, 21, 22, 24, GES-j = 1, 3, 4, 5, VIM-a = 1, 2, 4, 12, 19, IMP-b = 4, 8, CMY-a = 2, 4, 25, 31, 36, LAT-b = 1, 2, CMY-c = 1, 8b, 10, 19, FOX-d = 1, 5, 7, OXA-a = 1, 30, 47, OXA-2, OXA-9, OXA-b = 10, 17. Beta lactam antibiotics are classified into four classes, penams, cepheems, carbapenems and monobactams. One antibiotic name is listed as an example under each class. The beta lactamase, which can open the beta lactam ring is

indicated by R. For example, carbapenem is inactivated by KPC. If it is desired to re-sensitise bacteria to carbapenem, the spacer sequence 5'-TTGTTGCTGAAGGAGTTGGG should be employed into spacer array to inactivate KPC genes. Note that the spacer sequence for CMY-a can also be employed for LAT-b cleavage. The example of spacer sequences are shown from 5' to 3' direction.

Figure 17. Shows a set of spacer sequences (SEQ ID NOs: 61-77) that encode 17 guide RNA molecules targeted against 154 different *bla* genes identified in the CARD database for *Klebsiella pneumoniae* (see Example 5). Candidate spacer sequences were identified to disrupt all the *Klebsiella pneumoniae* beta lactamase genes found in the CARD database. This table was created with the same method explained in the figure legend in Figure 16. The example of spacer sequences are shown from 5' to 3' direction.

15

Figure 18. Map of a modified Cas DNA-binding polypeptide, Cas9R. A genetic fusion between Cas9 and Tn3 resolvase. Resolvase and Cas9 are indicated by arrows. The direction of the arrowhead represents the transcription polarity. Functional domain names of Cas9 are shown in the boxes below Cas9 open arrow. This Cas9 is the endonuclease activity deficient mutant dCas9, with amino acid substitutions D10A in RuvCI domain, H840A in HNH domain (as described by Tsai et al. [2014, Nature Biotechnology **32**: 569–576]). A mutant Tn3 resolvase (as described by Proudfoot et al. [2011 PLoS ONE 6(4): e19537]) is fused to the N-terminus of this dCas9 via a 12 mer polypeptide linker. Positions of some of these substituted amino acid residues reducing the specificity of the recombination site are indicated by short vertical bars in the N terminal domain, residues 1-148, of the resolvase. The full list of these substitutions is: R2A, E56K, G101S, D102Y, M103I, Q105L, V107F, E132A, L135R. In the Cas9 regions of the fusion: RuvCI, II, III, HNH and PI (PAM interaction) domains are nuclease domains, REC1a and REC1b are recognition domains of repeat and anti-repeat RNA, respectively. REC2 domain does not have any contact to the protospacer-gRNA heteroduplex. Four CRISPR spacer sequences S1, S2, S3 and S4 are arrayed under the expression of one CRISPR leader sequence and are required to bring about the Cas9R-mediated recombination event by the mutant Tn3 resolvase leading to the deletion and re-ligation of the target sequence. Tn3R = Tn3 Resolvase, R = Direct repeat, L = Leader sequence.

35

Figure 19. Schematic showing site-specific positioning of resolvase by gRNA directed Cas9. The open arrow in step I is the target antibiotic resistance gene on the plasmid for inactivation. Each recombination site A (A1, A2) and B (B1, B2) are recognised by gRNA independently and correctly positioned resolvases are dimerised in close proximity (step II).

- 5 Dimers in each recombination site A1+A2 and B1+B2 are tetramerised to form a synapse (step III). The synaptic complex (III) is enlarged, gRNAs are presented as dotted arrows designated S1, S2, S3 and S4. Large ovals represent dCas9, longitudinal ovals are resolvases connected via linker peptides. White and grey longitudinal ovals are resolvase catalytic domains dimerising on the recombination site B and A, respectively. The vertical
- 10 arrows indicate the cleavage position on the recombination sites by resolvase. The thin horizontal parallel arrows represent DNA containing the recombination site A1+A2 and the thick horizontal parallel arrows represent DNA containing the recombination sites B1+B2. The arrowhead shows the 3' end of the DNA sequence. Short black block arrows are locations of each of the PAM sequences.

15

Figure 20. Schematic showing exchanging half site of the recombination site A1+A2 and B1+B2 followed by strand resolution and sealing break point. Half-site of

- 20 recombination A1 and B1 are exchanged and ligated and resolved. The region of the target antibiotic gene is resolved as a circular DNA, while the rest of the chromosomal or plasmid replicon is re-circularised (step IV). Short black block arrows are locations of each PAM sequences after resolution.

- 25 **Figure 21.** Shows a set of spacer sequences (SEQ ID NOs: 78-85) that encode 8 guide RNA molecules targeted against the class A genes, SHV-a, CTX-M-b, TEM-c, KPC-d; the class B genes VIM-e, IMP-f, NDM-g and the class D gene, OXA-48 where SHV-a = 1, 1a, 2, 2a, 5, 5a, 11, 12, 14, 18, 20, 21, 22, 23, 26, 27, 28, 31, 32, 33, 38, 43, 44, 48, 52, 55, 56, 60, 61, 62, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 85, 89, 92, 98, 99, 100, 101, 103, 106, 107,
- 30 108, 109, 110, 111, 121, 136, 134, 137, 140, 143, 144, 147, 148, 149, 150, 151, 152, 153, 154, 155, 157, 158, 159, 160, 161, 162, 163, 164, 165, 168, 172, 173, 178, 179; CTXM-b = 1, 3, 10, 12, 15, 19, 22, 32, 52, 54, 59, 60, 62, 68, 71, 80, 81, 99, 141, 147; TEM-c = 1, 1B, 3, 139, 162, 183, 192, 197, 198, 209; KPC-d = 1, 2, 3, 4, 6, 7, 8, 11, 12, 14, 15, 16, 17 ; VIM-e = 1, 2, 4, 19, 26, 27, 33, 34; IMP-f = 4, 8, 32, 38; and NDM-g = 1, 9, 10 (see Example 7).

35

Figure 22. Shows a table giving the sequences (SEQ ID NOs: 86-98) of the oligonucleotides used in the construction of plasmids pNB200, 202, 203, 104A, 104B and 108 (see Example 7).

5

Figure 23. Plasmid map of pNB104A constructed in Example 7. The plasmid map was drawn by SnapGene viewer ver. 2.4.3 free version (<http://www.snapgene.com>). The tetramer spacer concatemer (Figure 29A) was digested with *BsaI*, whose restriction site is located in A1 and A2, and ligated to *BsaI* spacer cloning sites on pNB202 to give pNB203. The single promoter and spacer region (6221-7001) on pNB104A is shown. P = Promoter, L = Leader, R = Direct repeat, S = Spacer, T = Tail. The concatenated spacers (targeted against NDM, IMP, VIM and KPC) are located downstream of the single promoter.

Figure 24. Plasmid map of pNB104B constructed in Example 7. The plasmid map was drawn by SnapGene viewer ver. 2.4.3 free version (<http://www.snapgene.com>). The single promoter regions (6221-6987) on pNB104B is shown. P = Promoter, L = Leader, R = Direct repeat, S = Spacer, T = Tail. The concatenated spacers (targeted against OXA-48, SHV, TEM and CTX-M) are located under expression from the single promoter.

20

Figure 25. Plasmid map of pNB108 constructed in Example 7. The plasmid map was drawn by SnapGene viewer ver. 2.4.3 free version (<http://www.snapgene.com>). The octamer spacer concatemer (Figure 29B) was digested with *BsaI*, whose restriction site is located in A1 and A2, and ligated to *BsaI* spacer cloning sites on pNB100 to give pNB108. The single promoter and spacer region (6221-7225) on pNB108 is shown. P = Promoter, L = Leader, R = Direct repeat, S = Spacer, T = Tail. The concatenated spacers (targeted against NDM, IMP, VIM, KPC, OXA-48, SHV, TEM and CTX-M) are located under the single promoter.

30

Figure 26. Plasmid map of pNB200 constructed in Example 7. The plasmid map was drawn by SnapGene viewer ver. 2.4.3 free version (<http://www.snapgene.com>). The dual promoter cassette was synthesised by PCR from the template pNB100 with primer pair NB018 and NB019, the amplicon was digested with *BbvI* and ligated to the *BsaI* site of pNB100 to give pNB200, the small *BsaI* fragment of pNB100, from position 5776-5741 (see Figure 12) is replaced in the process. The dual promoter and two spacer cloning region (6221-7382) on pNB200 is shown. P = Promoter, L = Leader, R = Direct repeat, S = Spacer,

T = Tail. The restriction enzymes *Bsal* and *SapI* are utilised to clone upstream and downstream spacer sequences, respectively.

- 5 **Figure 27. Plasmid map of pNB202 constructed in Example 7.** The plasmid map was drawn by SnapGene viewer ver. 2.4.3 free version (<http://www.snapgene.com>). The tetramer spacer concatemer (Figure 29A) was digested with *SapI*, whose restriction site is located in B1 and B2, and ligated to *SapI* spacer cloning sites on pNB200 to give pNB202. The dual promoter and spacer regions (6221-7329) on pNB202 is shown. P = Promoter, L = Leader, R = Direct repeat, S = Spacer, T = Tail. The concatenated spacers (targeted against OXA-48, SHV, TEM and CTX-M) are located downstream of the second promoter.

- 15 **Figure 28. Plasmid map of pNB203 constructed in Example 7.** The plasmid map was drawn by SnapGene viewer ver. 2.4.3 free version (<http://www.snapgene.com>). The tetra spacer concatemer a+b+c+d shown in Figure 29A was digested with *Bsal*, whose restriction site is located in A1 and A2, and ligated to *Bsal* spacer cloning sites on pNB202 to give pNB203. The dual promoter and spacer regions (6221-7501) on pNB203 is shown. P = Promoter, L = Leader, R = Direct repeat, S = Spacer, T = Tail. The concatenated spacers (targeted against NDM, IMP, VIM and KPC) are located downstream of the first promoter. The concatenated spacers (targeted against OXA-48, SHV, TEM and CTX-M) are located downstream of the second promoter.

- 25 **Figure 29A. Tetramer spacer concatenation in Example 7.** The numbers associating oligos are corresponding to the primer numbers listed in Figure 22. Oligos are pairwise annealed between 26 and 27, 28 and 34, 35 and 31, 32 and 36 via a, c, e and g unique spacer region (I), respectively and extended in individual tubes (II). Dimer concatemer from 26 and 27 concatenate spacer a and b. Dimer concatemer from 28 and 34 concatenate spacer b, c and d. Dimer concatemer from 35 and 31 concatenate spacer e and f. Dimer concatemer from 32 and 36 concatenate spacer f, g and h (II). Concatenated dimmers a+b and b+c+d, e+f and f+g+h are further hybridised via b and f spacer region, respectively and extended to concatenate four spacers a, b, c and d or e, f, g and h (III). The tetramer spacer concatemer e+f+g+h was digested with *SapI*, whose restriction site is located in B1 and B2, and ligated to *SapI* spacer cloning sites on pNB200 to give pNB202. Tetra spacer concatemer a+b+c+d was digested with *Bsal*, whose restriction site is located in A1 and A2, and ligated to *Bsal* spacer cloning sites on pNB202 to give pNB203.

a = 20 mer spacer for NDM, b = 20 mer spacer for IMP, c = 20 mer spacer for VIM, d = 20 mer spacer for KPC, e = 20 mer spacer for OXA-48, f = 20 mer spacer for SHV, g = 20 mer spacer for TEM, h = 20 mer spacer for CTX-M.

5

Figure 29B. Octamer spacer concatenation in Example 7. The tetramer spacer concatemer a+b+c+d and e+f+g+h were amplified with primer pair NB026 and NB029, NB030 and NB033, respectively (V), and hybridise tetra concatemer via spacer d region followed by extension to yield octamer spacer a+b+c+d+e+f+g+h. This octamer was

10 digested with *Bsal* and ligated to *Bsal*, whose restriction site is located in A1 and A2, and ligated to *Bsal* spacer cloning sites on pNB100 to give pNB108. a = 20 mer spacer for NDM, b = 20 mer spacer for IMP, c = 20 mer spacer for VIM, d = 20 mer spacer for KPC, e = 20 mer spacer for OXA-48, f = 20 mer spacer for SHV, g = 20 mer spacer for TEM, h = 20 mer spacer for CTX-M.

15

Figure 30. Photographs showing results of “Nemesis symbiotic activity” (NSA) according to an embodiment of the invention by bacterial cell mating (see Example 7).

Fig 30A, top left plate shows JA200 pNT3 x DH5α pNB100 in Ap100Cm35, while top right plate shows JA200 pNT3 x DH5α pNB102 in Ap100Cm35. Fig 30B shows NCTC13440 x DH5α pNB100, the top left plate and NCTC13353 x DH5α pNB100, the top right plate; and NCTC13440 x DH5α pNB104A the bottom left plate and NCTC13353 x DH5α pNB104B, the bottom right plate all in Ap100Cm35. Fig 30C shows JA200 pNT3 x DH5α pNB100 (5–), NCTC13440 x DH5α pNB100 (2–) and NCTC13353 x DH5α pNB100 (4–), top left plate; and JA200 pNT3 x DH5α pNB108 (5/8), NCTC13440 x DH5α pNB108 (2/8) and NCTC13353 x DH5α pNB108 (4/8), top right plate and JA200 pNT3 x DH5α pNB100 (5+), bottom plate, all in Ap100Cm35.

As used herein, the term “antibiotic” refers to a classical antibiotic that is produced by a microorganism that is antagonistic to the growth of other microorganisms and also encompasses more generally an antimicrobial agent that is capable of killing or inhibiting the growth of a microorganism, including chemically synthesised versions and variants of naturally occurring antibiotics.

The term “sufficiently complementary” means that the sequence identity of the spacer sequence and the target sequence is such that the RNA guide molecule comprising the spacer sequence is able to hybridise, preferably specifically and selectively, with the target sequence, thereby allowing for inactivation of the antibiotic resistance gene comprising the

target sequence via the CRISPR/Cas system described herein. For example, the spacer sequence may have at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity over its entire length with the target sequence.

- 5 The term “functional equivalent” as used herein refers to a polypeptide which is capable of the same activity as a Cas DNA-binding polypeptide (or, as used herein, a Cas nucleic acid-binding polypeptide). The “functional equivalent” may have the same qualitative biological property as the Cas DNA-binding polypeptide. “Functional equivalents” include, but are not limited to, fragments or derivatives of a native Cas DNA-binding polypeptide
- 10 and its fragments, provided that the equivalents have a biological activity in common with a corresponding native sequence polypeptide. Although structural identity is not necessarily required for common biological activity, in one aspect the functional equivalent may have at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity over its entire length with a Cas DNA-binding polypeptide, for
- 15 example Cas9 (Ferretti *et al*, 2001, PNAS, 98 No. 8: 4658-4663, Gene ID: 901176, Cas9 GI: 15675041; SEQ ID NO: 99).

- The term “Cas DNA-binding polypeptide” encompasses a full-length Cas polypeptide, an enzymatically active fragment of a Cas polypeptide, and enzymatically active derivatives of a
- 20 Cas polypeptide or fragment thereof. Suitable derivatives of a Cas polypeptide or a fragment thereof include but are not limited to mutants, fusions, covalent modifications of a Cas protein or a fragment thereof.

- The term “modified” Cas DNA-binding polypeptide encompasses Cas DNA-binding
- 25 polypeptides as defined above except that the DSB catalytic function of the polypeptide is replaced by a DNA sealing function due for example to the presence of a recombinase catalytic domain. Further features of such modified Cas DNA-binding polypeptides are described herein.

- 30 Sequence identity between nucleotide or amino acid sequences can be determined by comparing an alignment of the sequences. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position. Scoring an alignment as a percentage of identity is a function of the number of identical amino acids or bases at positions shared by the compared sequences. When
- 35 comparing sequences, optimal alignments may require gaps to be introduced into one or more of the sequences to take into consideration possible insertions and deletions in the sequences. Sequence comparison methods may employ gap penalties so that, for the same

number of identical molecules in sequences being compared, a sequence alignment with as few gaps as possible, reflecting higher relatedness between the two compared sequences, will achieve a higher score than one with many gaps. Calculation of maximum percent identity involves the production of an optimal alignment, taking into consideration gap
5 penalties.

Suitable computer programs for carrying out sequence comparisons are widely available in the commercial and public sector. Examples include MatGat (Campanella et al., 2003, BMC Bioinformatics 4: 29; program available from <http://bitincka.com/ledion/matgat>), Gap
10 (Needleman & Wunsch, 1970, J. Mol. Biol. 48: 443-453), FASTA (Altschul et al., 1990, J. Mol. Biol. 215: 403-410; program available from <http://www.ebi.ac.uk/fasta>), Clustal W 2.0 and X 2.0 (Larkin et al., 2007, Bioinformatics 23: 2947-2948; program available from <http://www.ebi.ac.uk/tools/clustalw2>) and EMBOSS Pairwise Alignment Algorithms (Needleman & Wunsch, 1970, supra; Kruskal, 1983, In: Time warps, string edits and
15 macromolecules: the theory and practice of sequence comparison, Sankoff & Kruskal (eds), pp 1-44, Addison Wesley; programs available from <http://www.ebi.ac.uk/tools/emboss/align>). All programs may be run using default parameters.

For example, sequence comparisons may be undertaken using the “needle” method of the
20 EMBOSS Pairwise Alignment Algorithms, which determines an optimum alignment (including gaps) of two sequences when considered over their entire length and provides a percentage identity score. Default parameters for amino acid sequence comparisons (“Protein Molecule” option) may be Gap Extend penalty: 0.5, Gap Open penalty: 10.0, Matrix: Blosum 62. Default parameters for nucleotide sequence comparisons (“DNA Molecule” option) may be Gap
25 Extend penalty: 0.5, Gap Open penalty: 10.0, Matrix: DNAsfull.

In one aspect of the invention, a sequence comparison may be performed over the full length of the reference sequence.

30 As used herein, the term “gene” refers to a DNA sequence from which a polypeptide is encoded or a non-coding, functional RNA is transcribed.

The term “antibiotic resistance gene” encompasses a gene, or the encoding portion thereof, which encodes a product or transcribes a functional RNA that confers antibiotic resistance.
35 For example, the antibiotic resistance gene may be a gene or the encoding portion thereof which contributes to any of the four resistance mechanisms described above. The antibiotic resistance gene may for example encode (1) an enzyme which degrades an antibiotic, (2) an

enzyme which modifies an antibiotic, (3) a pump such as an efflux pump, or (4) a mutated target which suppresses the effect of the antibiotic.

The term “polynucleotide” refers to a polymeric form of nucleotide of any length, for example
5 RNA (such as mRNA) or DNA. The term also includes, particularly for oligonucleotide
markers, the known types of modifications, for example, labels which are known in the art,
methylation, “caps”, substitution of one or more of the naturally occurring nucleotides with an
analog, internucleotide modifications, such as, for example, those with unchanged linkages,
e.g., methyl phosphates, phosphotriesters, phosphoamidates, carbamates, etc. and with
10 charged linkages.

The term “polypeptide” as used herein refers to a polymer of amino acids. The term does not
refer to a specific length of the polymer, so peptides, oligopeptides and proteins are included
within the definition of polypeptide. The term “polypeptide” may include post-expression
15 modifications, for example, glycosylations, acetylations, phosphorylations and the like.
Included within the definition of “polypeptide” are, for example, polypeptides containing one
or more analogues of an amino acid (including, for example, unnatural amino acids),
polypeptides with substituted linkages, as well as other modifications known in the art both
naturally occurring and non-naturally occurring.

20 The term “microorganism” encompasses prokaryotes such as bacteria and archaea (for
example, those belonging to the the Euryarchaeota and Crenarchaeota). Bacteria include
both Gram positive and Gram negative bacteria. Some species of clinically significant,
pathogenic fungi are included in the definition of microorganisms, for example members of
25 the genus *Candida*, *Aspergillus*, *Cryptococcus*, *Histoplasma*, *Pneumocystis* and
Stachybotrys.

Various aspects of the invention are shown in the figures and described below.

30 In the present invention, an advantage of using a bacteriophage or conjugative plasmid that
comprises the recombinant polynucleotide of the invention is that each serves as a “Trojan
horse” that, following infection of the bacteriophage, or following plasmid conjugation, results
in the insertion of the “assassin construct” into the target bacteria or other microorganism.

35 The assassin construct once inserted into the target microorganism also provides an
immunisation of the cells against the future arrival of a plasmid harbouring antibiotic

resistance genes (see Figure 2), in addition, of course to disrupting such genes already present (see Figures 1 and 3).

The assassin constructs then begin the process of degradation of the antibiotic resistance
5 genes. If a DSB created by the Cas DNA-binding protein of the invention destroys a replicon carrying such an antibiotic resistant gene then a microorganism harbouring the antibiotic resistance gene may be killed directly by an assassin construct. If the microorganism survives the DSB, the resistance gene will be inactivated, and a patient may then be treated with the antibiotic(s) to which the microorganism has now become sensitised.

10

Importantly, there should be no or reduced direct selection pressure acting against this event if and until patients are subsequently treated with antibiotics. Thus there should be little or no direct selection for bacteriophage (or plasmid) resistance in the pathogenic bacteria or other microorganisms and therefore no or less establishment of an “evolutionary arms race” –
15 sometimes a significant limiting feature of the known use of bacteriophage directly as bactericidal agents.

In the event that DSB-induced killing of a microorganism increases selection pressure for resistance to a bacteriophage or conjugative plasmid delivery agent, the problem could be
20 mitigated by, for example, using a modified Cas DNA polypeptide as defined herein.

This present invention provides potential agents for oral, topical and probiotic, dietary supplement delivery as well as an epidemiological tool to silently inactivate antibiotic resistance genes in pathogenic bacteria or other microorganisms (see Figure 3). Patients
25 scheduled for surgery, or other treatment in hospital, may well be treated with recombinant bacteriophage carrying CRISPR/Cas9 (or other) assassin constructs targeted against antibiotic resistance genes prophylactically in advance of hospital admission. In this way, pathogens present in their microbiome can be directly killed or purged of antibiotic resistance genes in anticipation of any post-operative infection that might occur requiring treatment with
30 antibiotics.

Thus this present invention provides an epidemiological tool to silently inactivate antibiotic resistance genes in pathogenic bacteria.

35 To effect exemplification of the invention, a set of CRISPR/Cas9 assassins targeted against selected antibiotics may be constructed. Variables are the bacteriophage (also referred to herein synonymously simply as “phage”), or conjugative plasmid, or DNA, delivery agent: a

range of bacteriophage and plasmid agents may be developed that are specific to a range of important bacterial pathogens.

The extent to which a single generalised bacteriophage or plasmid delivery agent needs to be modified to target different bacterial pathogens depends on the details of the specificity of the interactions of either the phage proteins involved in the phage life-cycle, or the plasmid biology and the pathogenic bacterial target.

Both lysogenic phage that infect hosts and become dormant as prophage, as well as non-virulent phage that replicate but do not kill the host, may be developed. With regards to lysogenic phage specificity, only the lysogenic life cycle of the phage and hence the specificity involved in (i) entry of the phage into the bacterial cell and (ii) its subsequent integration into an attachment site in the target chromosome is required. Integration may not be needed and may be replaced by using the phage to deliver a plasmid that can then excise, for example by cre-lox recombination, and replicate independently in the cell. Non-virulent M13 phage and derivatives thereof may be used.

A functional lytic cycle in lysogenic phage may be retained such that low levels of entry into the lytic cycle in lysogenised bacteria will generate new phage that can go on to subsequently infect other bacteria (either pathogenic bacteria or non-pathogenic bacteria, to provide immunity). From the point of view of the epidemiological spread of the phage in the pathogenic population this may not be necessary and a single initial infection may suffice. This can be tested experimentally. Optimal conditions for efficient infection and the appropriate multiplicity of infection are identified.

Experiments may be performed in a model system (see Figure 1). For example, *E. coli* carrying multiple drug resistance, for example, to ampicillin, chloramphenicol, and kanamycin or targeted against commonly used antibiotics against which resistance is widespread.

Experiments may also be performed with a target pathogen, for example, *Klebsiella pneumoniae* carbapenemase ("KPC"). KPC is responsible for infection and death in hospitals in the UK.

Delivery by bacteriophage: The phage delivery system (see for example Figure 2, route 2) may be suitable for the treatment of wounds and burns infected by antibiotic-resistant bacteria.

Delivery of the assassin construct targeted against these antibiotics by two different *E.coli* lysogenic bacteriophage: bacteriophage lambda and bacteriophage Mu may be used.

- Bacteriophage lambda has a specific integration site in the host chromosome known as the
 5 lambda attachment site. Bacteriophage Mu is able to integrate randomly into the host genome and has the advantage that no specific attachment sites in bacteria are required.

- Phage may also be used to deliver a plasmid replicon containing the recombinant polynucleotide of the invention that excises by cre-lox recombination following infection as
 10 discussed above or by use of phage P1 that replicates as an episome. The specificity involved in successful infection is recognition of a membrane protein on the bacterial cell surface. In the case of lambda this is the maltose permease protein that transports the sugar maltose into the bacterial cell. In the case of bacteriophage Mu the receptor is LPS.

- 15 Male-specific phage M13 that infect *E.coli* cells carrying the F-factor plasmid may also be used to deliver CRISPR/Cas9 constructs (or other assassin constructs of the invention) targeted against one or more antibiotic resistance genes. M13 is a well studied phage, which replicates, but does not kill the bacterial host.

- 20 Successful elimination of resistance to target antibiotics following the lyogenisation by recombinant bacteriophage, or infection by M13 recombinants carrying the assassin construct directed against these antibiotic resistance genes is evaluated.

- Studies on other closely related Enterobacteriaceae carrying resistance to the same
 25 antibiotics may be performed. These may include *Salmonella typhimurium*, and *Shigella flexneri* in addition to *Klebsiella pneumoniae* discussed above.

- In order to do this, modified phage are constructed with different genes encoding the tail fibre protein of the bacteriophage in order to allow it to interact with a different receptor present on
 30 the bacterial surface. Lysogenic phage, or male-specific phage, like M13, that are natural hosts of these bacteria may be used.

- The phenomenon of phase variation whereby bacteriophage are able to switch their host specificity by the inversion, at a low frequency, of a DNA segment to control gene expression
 35 and express alternative tail fibre proteins may also be exploited. And in that way develop phage delivery systems that are versatile. For example phage Mu carries an invertible G segment (regulated by a Mu-encoded site-specific recombinase, Gin) giving rise to two

phage types G(+) able to infect *E.coli* K12 and G(–) able to infect *Enterobacter cloacae*, *Citrobacter freundii* *Serratia marcescens* and *Erwinia carotovora*.

Another example is the *Neisseria gonorrhoeae* filamentous phage NgoPhi6, or modified
 5 forms thereof. The natural (wild type) phage has a wide host range for Gram negative
 bacteria (alpha, beta and gamma proteobacteria) – see Piekarowicz et al. (2014 J. Virol. 88:
 1002 - 1010). The natural phage is not lytic but lysogenic and has an integration site on the
 host genome. In one aspect of the invention, the ability of the phage to integrate into the host
 chromosome is removed and the phage is engineered to replicate independently. The
 10 substitution of the left integration site L, CTTATAT with L', CATATAT will eliminate the
 integration event. In order to replicate the phage genome extra-chromosomally, the M13 ori
 and M13 gene II can be used to mimic the M13 phage replication. The modification may be
 kept to a minimum to maintain the ability of the progeny production from the infected
 bacteria. Although the maximum packaging size is unknown, a phage DNA of around 12 Kb
 15 is experimentally demonstrated as packageable and phage progeny produced from the
 bacteria infected with this phage are infectable. The length of the phage is around 4 microns,
 which is 4.4 times longer than M13 phage particle, and indicates the higher packaging
 capacity of DNA longer than M13 – i.e. assuming that the packaging size of DNA is
 proportional to the size of the phage particle length, DNA packaging capacity of the NgoPhi6
 20 phage will $4 \text{ microns} / 0.9 \text{ microns} \times 6407 \text{ nt} = 28475 \text{ nt}$, large enough for packaging phage
 genome along with a recombinant polynucleotide of the invention, including for example a
 CRISPR-Cas9 construct if required. The exemplified structure of the phage vector
 (phagemid) meets the requirements – see Figure 15.

25

Delivery by conjugative plasmids: Delivery of the assassin constructs targeted against
 these antibiotics by selected broad-host range conjugative plasmids may be evaluated (see
 Figure 4, route 3). Here, the plasmids may be delivered by a benign non-pathogenic host.
 The application here may be for the GI tract as a probiotic and may be used prophylactically.
 30 In this aspect, the possible lethal effects of DSBs caused by Cas DNA-binding protein are
 not a concern since the plasmid will not be transferred to the recipient.

Similarly demonstration of the successful elimination of resistance to the three target
 antibiotics following conjugal transfer of plasmids carrying the assassin cargo may be
 35 evaluated.

Delivery by transformation of linear DNA: Delivery of the assassin construct targeted against these antibiotics by linear double-stranded DNA transformation (see Figure 4, route 1) may also be performed. Here, the DNA is delivered via DNA receptor on the surface of the bacteria from the bacterial surrounding environment.

5

Examples

The present invention is illustrated by the following non-limiting examples.

5 Example 1

The aim of Example 1 is to demonstrate a proof-of-concept for resurrection of antibiotic efficacy by introduction of a CRISPR/Cas9 construct in non-pathogenic bacterial strains of *Escherichia coli*.

- 10 A CRISPR/Cas9 construct is made directly from genomic DNA isolated from the *Streptococcus pyogenes* strain SF370, which is obtained from:
<http://www.straininfo.net/strains/117800/browser.jsessionid=A07A638D6D2472EA2FEDBD3A1928F347.straininfo2>. Here the Cas9 coding region plus adjacent regulatory regions and DNA encoding the tracrRNA is extracted by PCR, using sequence-specific DNA primers and
15 cloned into a suitable plasmid cloning vector. A unit repeat of CRISPR array comprising the direct repeats flanking a spacer sequence is similarly extracted by PCR and modified to replace the spacer sequence by a cloning site to allow the subsequent introduction of spacer sequences designed to target DNA regions of choice such as antibiotic resistance genes. It is useful to have a positive selection for bacterial transformants carrying the desired
20 recombinants in which such a spacer sequence has been successfully cloned into the cloning site. Appendix 3 gives an example of such a positive selection.

- Alternatively an equivalent CRISPR/Cas9 gene targeting construct, though lacking a positive selection for recombinants, is obtained from a pCas9 plasmid such as for example available
25 from Addgene: <http://www.addgene.org/42876/>. This plasmid carries the Cas9 gene plus a DNA sequences encoding tracrRNA and CRISPR array with a unique cloning site in order to introduce the spacer sequence desired to target a given DNA sequence for cleavage by the Cas9 endonuclease. For exemplification purposes, use of this existing pCas9 plasmid is described below.

- 30 Example 1 shows that bacteria carrying a beta-lactamase (*bla*) gene conferring resistance to the beta-lactam antibiotic, ampicillin become sensitive to ampicillin following introduction of a modified CRISPR/Cas9 construct targeted against the *bla* gene: the CRISPR/Cas9/anti-*bla* construct.

35

Materials and Methods

A. Bacterial strains, plasmids and phage

1. Bacterial strains:

5 DH5 α is (F⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ 80d/lacZ Δ M15 Δ (lacZYA-argF)U169, hsdR17(r_K⁻ m_K⁺), λ -).

JM109 is (endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB⁺ Δ (lac-proAB) e14- [F' traD36 proAB⁺ lacI^q lacZ Δ M15] hsdR17(r_K⁻ m_K⁺)).

10 2. Plasmids:

pUC18 (*Ori* pMB1); pCas9 (pACYC184-based vector with *Ori* p15A, CRISPR locus plus Cas9 gene, CM^R); pCRISPR (*Ori* pMB1, CRISPR, Kn^R).

3. Phage: M13mp18

15

In Example 1A, in one strain *E.coli* DH5 α and carrying pBR322 a medium copy plasmid or alternatively a low copy plasmid, the CRISPR/Cas9/anti-*bla* construct is delivered, by naked DNA transformation in plasmid pCas9, designated pCas9::anti-*bla*. The already present plasmid pBR322, or the low copy plasmid expresses the beta-lactamase derived from
20 bacterial transposon Tn3; pBR322 also carries resistance to tetracycline. As pCas9::anti-*bla* carries resistance to chloramphenicol, selection for cells maintaining pCas9::anti-*bla* is achieved by addition of chloramphenicol to the growth medium. In a separate negative control experiment DH5 α cells with pBR322, or a low copy plasmid are transformed with pCas9 (that is a plasmid in all respects like pCas9::anti-*bla* but lacking anti-*bla* which is the
25 spacer sequence targeted against the *bla* gene: it is predicted that pCas9, in lacking the anti-*bla* would not be able to attack and inactivate the *bla* gene. Again pCas9 is maintained by the presence of chloramphenicol.

Beta-lactamase activity can be detected by nitrocefin, which is a chromogenic derivative of
30 cephalosporin, when the beta-lactam ring is hydrolysed, ultraviolet absorption of intact nitrocefin is shifted to around 500 nm, which allows visual detection of the presence of beta lactamase.

Let the total number of bacteria resistant to chloramphenicol be N_0 and the number of beta-
35 lactamase resistant bacteria be N_r . Growth of N_r manifest as red colonies in the presence of nitrocefin. Let the number of beta lactamase sensitive bacteria be N_w . Growth of N_w colonies manifest as white colonies in the presence of nitrocefin. Thus the efficacy of

CRISPR/Cas9/anti-*bla*-mediated inactivation of the *bla* gene efficiency is calculated by measuring the fraction of white colonies, i.e. $N_w/N_0 = (N_0 - N_r)/N_0$.

Alternatively beta lactamase activity is seen directly challenging the bacteria on the LB plate containing ampicillin. Total bacteria resistant to chloramphenicol is N_0 , ampicillin resistant bacteria is N_a , then CRISPR/Cas9/anti-*bla*-mediated inactivation of the *bla* gene efficiency can be defined by measuring the fraction of the ampicillin sensitive colony, $1 - N_a/N_0$.

In Example 1B, an M13 phage delivery system is used to introduce CRISPR/Cas9/anti-*bla* construct into the *E. coli* strain, JM109, expressing beta lactamase gene from a resident plasmid.

M13::CRISPR/Cas9/anti-*bla* phage recombinant is prepared as follows: the CRISPR/Cas9/anti-*bla* construct is isolated from pCas9::anti-*bla* by digesting this construct with *Sa*I and *Xba*I. The digested fragment size is 5796 bp. The fragment is cloned at *Sa*I (GTCGAC) and *Xba*I (TCTAGA) site of M13mp18 RF I. The entire size of the M13mp18 containing CRISPR/Cas9/ anti-*bla* construct is 13035 bp. This M13 recombinant phage DNA is transformed to *E. coli* JM109, a bacterial strain carrying the F' plasmid and recombinant M13 phage extruded from the bacteria is purified and used to introduce the CRISPR/Cas9 construct to *E. coli* JM109 harbouring pBR322. As a negative control, an equivalent M13::CRISPR/Cas9 phage recombinant lacking anti-*bla* region is prepared from pCas9 by restriction enzyme *Sa*I and *Xba*I and the amplicon is cloned at *Sa*I and *Xba*I site of M13mp18 RF I.

When M13 infects the bacterial cells, it does not kill them, but the phage DNA replicates inside the cell and expresses phage genes. Thus M13 phage infection with an M13::CRISPR/Cas9/anti-*bla* phage recombinant should result in inactivation of the *bla* gene, in contrast, in a negative control, to infection by an M13::CRISPR/Cas9 phage recombinant lacking anti-*bla* region. Because M13 phage infection slows down the rate of cell growth, when infected cells grown on a lawn of cells yield turbid plaques of slow growing infected cells. If nitrocefin is added to the growth medium, the efficacy of CRISPR/Cas9/anti-*bla*-mediated inactivation of the *bla* gene efficiency is calculated by measuring the fraction of white colonies to red colonies, when plaques are picked and grown as colonies to remove the background lawn. Plaques may also be picked and plated onto LB plate with/without ampicillin to score the ratio of ampicillin sensitive to ampicillin resistant colonies that will result.

B. Selection of spacer sequence from the target sequence

We can use Guide RNA Target Design Tool (see;

<https://www.blueheronbio.com/external/tools/gRNASrc.jsp>) from BlueHeron to select spacer sequence from the target. This program simply returns the 20 nt spacer sequence with the

- 5 appropriate PAM (protospacer adjacent motif) sequence in the 3' end and GC content. It does not consider secondary structure stability and sequence specificity. Secondary structure prediction and specificity search is performed manually.

- We choose the actual spacer sequence from the candidate sequences obtained in the above
- 10 program, which should meet the following two criteria: 1) low tendency to form stable secondary structure of crRNA, 2) no target DNA on the host genomic DNA. It may be very difficult to find a unique sequence to satisfy criterion No.2. Considering mismatched target data from Figure 3 E in Jinek *et al*/ Science 337, 816 (2012), criterion No.2 is relaxed to allow a matched sequence up to the 12th nucleotide position in the target sequence (the first
- 15 nucleotide position is counted from just next to the PAM sequence). In other words, when the first 12 mer protospacer sequence of the target sequence is completely matched to the 12 mer sequence of crRNA spacer sequence in the 3' end, but the rest of the sequence is not matched, it is assumed that target dsDNA is not cleaved. The specificity check of the protospacer sequence along the *E. coli* K12 genome sequence is performed by BLAST. The
- 20 *b/a* sequence is searched against the subject sequence *Escherichia coli* str. substr. MG1655 ([http://www.ncbi.nlm.nih.gov/nucleotide/556503834?report=genbank&log\\$=nuclalign&blast_rank=1&RID=JUYB76FX014](http://www.ncbi.nlm.nih.gov/nucleotide/556503834?report=genbank&log$=nuclalign&blast_rank=1&RID=JUYB76FX014)), and each of any matched chromosomal sequence is mapped against the *b/a* sequence for counting the seed sequence from the canonical PAM (NGG) sequence. Secondary structures can be predicted by mFold.
- 25 (<http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form>) to choose the sequence whose G is large as possible, preferably to be positive for crRNA spacer secondary structure. The following is the way to select the appropriate spacer sequences from the *b/a* sequence.

Seq	Location	anti-protospacer seq	SEQ ID NO	MG1655	pCas9	pBR322	PAM	GC	ΔG
				Off targ	Off targ	Off targ			
05	83-102 (F)	TAGATAACTACGATACGGGA	100	CGGGA	None	None	GGG	0.48	-0.40
05	83-102 (F)	TAGATAACTACGATACGGGA	100	5			GGG	0.48	+0.50
22	442-461 (F)	GATCGTTGTCAGAAGTAAGT	101	AGTAAGT	AGTAAGT	None	TGG	0.43	-0.80
22	442-461 (F)	GATCGTTGTCAGAAGTAAGT	101	7	7		TGG	0.43	-0.50

30	647- 666 (F)	ACTTTAAAAGTGCTCATCAT	102	TCAT	ATCAT	None	TGG	0.35	-1.30
30	647- 666 (F)	ACTTTAAAAGTGCTCATCAT	102	4	5		TGG	0.35	-1.30
35	767- 786 (F)	TTTACTTTCACCAGCGTTTC	103	GCGTTTC	TTTC	None	TGG	0.43	+1.30
35	767- 786 (F)	TTTACTTTCACCAGCGTTTC	103	7	4		TGG	0.43	+2.20
65	231- 250 (RC)	ATTAATAGACTGGATGGAGG	104	GATGGAGG	GAGG	GAGG	CGG	0.48	+0.00
65	231- 250 (RC)	ATTAATAGACTGGATGGAGG	104	8	4	4	CGG	0.48	+0.90
66	234- 253 (RC)	ACAATTAATAGACTGGATGG	105	GATGG	None	None	AGG	0.39	-0.30
66	234- 253 (RC)	ACAATTAATAGACTGGATGG	105	5			AGG	0.39	+0.20
67	237- 256 (RC)	GCAACAATTAATAGACTGGA	106	GACTGGA	CTGGA	CTGGA	TGG	0.39	-0.20
67	237- 256 (RC)	GCAACAATTAATAGACTGGA	106	7	5	5	TGG	0.39	-0.20
68	241- 260 (RC)	CCCGGCAACAATTAATAGAC	107	AATAGAC	AGAC	None	TGG	0.48	+1.80
68	241- 260 (RC)	CCCGGCAACAATTAATAGAC	107	7	4		TGG	0.48	+2.60
69	259- 278 (RC)	AACTACTTACTCTAGCTTCC	108	AGCTTCC	GCTTCC	None	CGG	0.48	+0.60
69	259- 278 (RC)	AACTACTTACTCTAGCTTCC	108	7	6		CGG	0.48	+1.60
70	284- 303 (RC)	ACGTTGCGCAAACTATTAAC	109	TATTAAC	None	TAAC	TGG	0.43	-1.90
70	284- 303 (RC)	ACGTTGCGCAAACTATTAAC	109	7		4	TGG	0.43	-1.90
73	375- 394 (RC)	TGTAACCTCGCCTTGATCGTT	110	TCGTT	CGTT	CGTT	GGG	0.52	-0.50
73	375- 394 (RC)	TGTAACCTCGCCTTGATCGTT	110	5	4	4	GGG	0.52	+0.10
74	376- 395 (RC)	ATGTAACCTCGCCTTGATCGT	111	TTGATCGT	TCGT	None	TGG	0.48	-0.50
74	376- 395 (RC)	ATGTAACCTCGCCTTGATCGT	111	8	4		TGG	0.48	+0.10
81	443- 462 (RC)	AACTTACTTCTGACAACGAT	112	CAACGAT	None	None	CGG	0.43	+0.40
81	443- 462 (RC)	AACTTACTTCTGACAACGAT	112	7			CGG	0.43	+0.40
84	528- 547 (RC)	AGTCACAGAAAAGCATCTTA	113	GCATCTTA	None	None	CGG	0.43	-0.50
84	528- 547 (RC)	AGTCACAGAAAAGCATCTTA	113	8			CGG	0.43	+0.50
90	638- 657 (RC)	ACTTTTAAAGTTCTGCTATG	114	GCTATG	None	None	TGG	0.35	-0.70
90	638- 657 (RC)	ACTTTTAAAGTTCTGCTATG	114	6			TGG	0.35	+0.10

Table 1. Candidate anti-protospacer sequence.

All the target sequence from the *b/a* gene was obtained using Guide RNA Target Design Tool (<https://www.blueheronbio.com/external/tools/gRNASrc.jsp>) from BlueHeron. There are 98 target candidate sequences returned. Bacterial off-target chromosomal short similar

sequences are mapped against the *b/a* gene followed by counting the seed sequence from the canonical PAM sequence. Choose the sequences whose seed sequence number is less than eight and whose Gibbs free energy is relatively large. The summary of the property of the selected target sequences is shown in Table 1. This table also shows

5 nucleotide length of the seed sequence of the off-target sequences on pCas9 and pBR322.

Oligo cassette sequence for spacer sequence

The following four spacer sequences are crRNA generating cassettes targeting beta-lactamase on pBR322 in *E. coli* as a host strain, which meet the above two criteria. crRNA CR05 cleaves phosphodiester bond between 762nd base C and 763rd base C, CR30

10 cleaves phosphodiester bond between 198th base G and 199th base A, CR70 cleaves phosphodiester bonds between 575th base T and 576th base A and CR90 cleaves phosphodiester bonds between 221st base T and 222nd base A on the beta-lactamase gene.

15 Adaptors for single targets on the beta-lactamase gene.

CR05

5' -AAACTAGATAACTACGATACGGGA_g SEQ ID NO: 115
atctattgatgctatgccctcAAAA-5' SEQ ID NO: 116

20

CR30

5' -AAACACTTTTAAAGTGCTCATCAT_g SEQ ID NO: 117
tgaaattttcacgagtagtacAAAA-5' SEQ ID NO: 118
DraI

25

CR70

5' -AAACACGTTGCGCAAACCTATTAAC_g SEQ ID NO: 119
tgcaacgcgtttgataattgcAAAA-5' SEQ ID NO: 120
AclI

30

CR90

5' -AAACACTTTTAAAGTTCTGCTATG_g SEQ ID NO: 121
tgaaaatttcaagacgataaccAAAA-5' SEQ ID NO: 122
DraI

35

Adaptor for dual targets on the beta-lactamase gene.

Internal direct repeat sequence is italicised and underlined.

CR30+CR90

SEQ ID NO: 123

40 5' -AAACACTTTTAAAGTGCTCATCATGTTTGTAGAGCTATGCTGTTTGAATGGTCCCAAAACACTTTTAAAGTTCTGCTATG_g
tgaaattttcacgagtagtacaaaaatctcgatacgacaaaacttaccagggttttgtaaaatttcaagacgataaccAAAA
DraI CR30 *DraI* CR90

SEQ ID NO: 124

The 5' end of each oligo is phosphorylated and ready for cloning at *Bsa*I sites.

Sites of six base cutter restriction endonucleases are underlined, which are useful to screen the recombinants. We can also employ one of the cassette oligos as a primer to screen the recombinants by PCR together with another unique primer for the plasmid vector.

5

Appendix 1

pCas9 plasmid sequence (SEQ ID NO: 125)

Cas 9 gene, CRISPR expression locus and tracrRNA (all from *S. pyogenes*)

10

GAATTCGGATGAGCATTATCAGGCGGGCAAGAAATGTGAATAAAGCCGGATAAACTTGTGCTTATTTTCTTTACGG
TCTTTAAAAAGCCGTAATATCCAGCTGAACGGTCTGGTTATAGGTACATTGAGCAACTGACTGAAATGCCTCAAAATGT
TCTTTACGATGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCATTTTAGCTTCCTTAGCTCCTGA
AAATCTCGATAACTCAAAAAATACGCCGGTAGTGATCTTATTTTCAATTATGGTGAAAGTTGGAACCTCTTACGTGCCGAT
15 CAACGCTCATTTTTGCGCAAAAGTTGGCCAGGGCTTCCCGGTATCAACAGGGACACCAGGATTTATTTATCTGCGAAG
TGATCTTCCGTCACAGGTATTTATTCGGCGCAAAGTGCCTGGGTGATGCTGCCAACTTACTGATTTAGTGATGATGGT
GTTTTTGGGTGCTCCAGTGGCTTCTGTTTCTATCAGCTGTCTCTCTGTTTCTGCTACTGACGGGGTGGTGCGTAACGGC
AAAAGCACCGCCGGACATCAGCGCTAGCGGAGTGATACTGGCTTACTATGTTGGCACTGATGAGGGTGTCTAGTGAAGTG
CTTCATGTGGCAGGAGAAAAAGGCTGCACCGGTGCGTCAGCAGAAATATGTGATACAGGATATATTCCGCTTCTCTCGCTC
20 ACTGACTCGCTACGCTCGGTCTTCGACTGCGGCGAGCGGAAATGGCTTACGAACGGGGCGGAGATTTCTGGAAGATGC
CAGGAAGATACCTTAACAGGGAAGTGAGAGGGCGCGGCAAAGCGTTTTTCCATAGGCTCCGCCCCCTGACAAGCATCA
CGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGCGGCTCCC
TCGTGCGCTCTCTGTTCTCTGCTTTCCGTTTACCGGTGTCATTCCGCTGTTATGGCCGCGTTTGTCTCATTCCACGCCT
GACACTCAGTTCCGGGTAGGCAGTTCTGCTCCAAGCTGGACTGTATGCAGAACCCCCCTTCACTCCGACCGCTGCGCCT
25 TATCCGGTAACATATCGTCTTGAGTCCAACCCGGAAAGACATGCAAAAGCACCACTGGCAGCAGCCACTGGTAATTGATTT
AGAGGAGTTAGTCTTGAAGTCATGCGCCGGTTAAGGCTAAACTGAAAGGACAAGTTTTGGTGACTGCGCTCCTCCAAGCC
AGTTACCTCGGTTCAAAGATTGGTAGCTCAGAGAACCTTCGAAAAACCGCCCTGCAAGGCGGTTTTTTCGTTTTTCAGAG
CAAGAGATTACGCGCAGACCAAAACGATCTCAAGAAGATCATCTTATTAATCAGATAAAAATATTCTAGATTTCAGTGCA
ATTTATCTCTTCAAATGTAGCACCTGAAGTCAGCCCCATACGATATAAGTTGTAATTCTCATGTTTGACAGCTTATCATC
30 GATAAGCTTTAATGCGGTAGTTTATCACAGTTAAATGCTAACGCAGTCAGGCACCGGTATGAAATCTAACAATGCGCT
CATCGTCATCTTCGGCACCGCTCACCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCTCTTGGCGGG
ATTACGAAATCATCTCTGTGAGCTTAGTAGGTTTAGCAAGATGGCAGCGCCTAAATGTAGAATGATAAAGGATTAAGAG
ATTAATTTCCCTAAAAATGATAAAACAAGCGTTTTGAAAGCGCTTGTTTTTTGGTTTGCAGTCAGAGTAGAATAGAAGT
ATCaaaaaaagcaccgactcggtgccactttttcaagttgataacggactagccttattttaacttgctatgctggtttt
35 aatggttccacaagattatTTTATAACTTTTATAACAAATAATCAAGGAGAAATTCAAAGAAATTTATCAGCCATAAAA
CAATAGATTAACTATAGAATGATAACAAAAATAAATCTTTTAAAGAAATTTGTGTTATAATCTATTTATTTAAG
TATTGGGTAATATTTTTTGAAGAGATATTTTGAAGAAAGAAAAATTAAGCATATTAACATAATTTCCGAGGTCATTA
CTATTATTGAAATCATCAAACCTATTATGATTAAATTTAACTTTTTATTTTAGGAGGCAAAA

40

ATGGATAAGAAATCTCAATAGGCTTAGATATCGGCACAAATAGCGTCGGATGGGCGGTGATCACTGATGAATATAAGGT
TCCGCTCAAAAAGTTCAAGGTTCTGGGAAATACAGACCGCCACAGTATCAAAAAAATCTTATAGGGGCTCTTTTATTTG
ACAGTGGAGAGACAGCGGAAGCGACTCGTCTCAAACGGACAGCTCGTAGAAGGTATACACGTCGGAAGAATCGTATTTGT
TATCTACAGGAGATTTTTCAAATGAGATGGCGAAAGTAGATGATAGTTTCTTTTCACTGACTTGAAGAGTCTTTTTTGGT
45 GGAAGAAGACAGAAGCATGAACGTCATCCTATTTTTTGGAAATATAGTAGATGAAGTTGCTTATCATGAGAAATATCCAA
CTATCTATCATCTGCGAAAAAATGGTAGATTCTACTGATAAAGCGGATTTGCGCTTAATCTATTTGGCCTTAGCGCAT
ATGATTAAAGTTTCGTGGTCATTTTTTGATTGAGGGAGATTTAAATCCTGATAATAGTGATGTGGACAACTATTTATCCA
GTTGGTACAAACCTACAATCAATTATTTGAAGAAAACCTATTAACGCAAGTGGAGTAGATGCTAAAGCGATCTTTCTG
CACGATTGAGTAAATCAAGACGATTAGAAAATCTCATTTGCTCAGCTCCCCGGTGAGAAGAAAAATGGCTTATTTGGGAAT
50 CTCATTGCTTTTGTCTTTGGGTTTGACCCCTAATTTTAAATCAAATTTTGATTGGCAGAAAGATGCTAAATTACAGCTTTC
AAAAGATACTTACGATGATGATTTAGATAATTTATTGGCGCAAATTTGGAGATCAATATGCTGATTTGTTTTGGCAGCTA
AGAATTTATCAGATGCTATTTTACTTTTCAAGATATCCTAAGAGTAAATACTGAAATACTAAGGCTCCCTATCAGCTTCA
ATGATTAAACGCTACGATGAACATCATCAAGACTTGACTCTTTTAAAGCTTTAGTTTCGACAACAACCTCCAGAAAAAGTA
TAAAGAAATCTTTTTTGTATCAATCAAAAAACGGATATGCAGGTTATATTGATGGGGGAGCTAGCCAAGAAGAAATTTATA
AATTTATCAAACCAATTTTAGAAAAATGGATGGTACTGAGGAATTATTTGGTGAACATAATCGTGAAGATTTGCTGCGC
55 AAGCAACGGACCTTTGACAACGCTCTATTCCCATCAAATTCACCTGGGTGAGCTGCATGCTATTTTGAAGACAAGA
GACTTTTTATCCATTTTTTAAAGACAATCGTGAGAAGATTGAAAAATCTTGACTTTTCGAATTCCTTATTTATGTTGGTCT

5 CATTGGCGCGTGGCAATAGTCGTTTTGTCATGGATGACTCGGAAGTCTGAAGAAACAATTACCCCATGGAATTTTGAAGAA
 GTTGTGCATAAAGGTGCTTCAGCTCAATCATTATTGAAACGCATGACAACTTTGATAAAAATCTTCCAAATGAAAAAGT
 ACTACCAAAACATAGTTTGCCTTATGAGTATTTTACGGTTTATAACGAATTGACAAAGGTCAAAATATGTTACTGAAGGAA
 TGCAGAAAACAGCATTTCTTTTCAAGGTGAACAGAAAGGCCATTGTTGATTTACTCTTCAAAACAAATCGAAAAGTAACC
 10 GTTAAGCAATTAAAAGAAGATTATTTCAAAAAATAGAATGTTTTGATAGTGTGAAATTTTCAAGAGTTGAAGATAGATT
 TAATGCTTCATTAGGTACCTACCATGATTTGCTAAAAATTAATAAGATAAAGATTTTGGATAATGAAGAAAATGAAG
 ATATCTTAGAGGATATGTTTTAACATTGACCTTATTTGAAGATAGGGAGATGATTGAGGAAAGACTTAAACATATGCT
 CACCTCTTTGATGATAAGGTGATGAAACAGCTTAAACGTCGCCGTTTACTGGTTGGGGACGTTTGTCTCGAAAATTGAT
 TAATGGTATTAGGGATAAGCAATCTGGCAAAACAATATTAGATTTTTTGAATCAGATGGTTTTGCCAAATCGCAATTTTA
 15 TGCAGCTGATCCATGATGATAGTTTGACATTTAAAGAAGACATTCAAAAGCACAAGTGTCTGGACAAGGCGATAGTTTA
 CATGAACATATTGCAAAATTTAGCTGGTAGCCCTGCTATTAAGAAAGGTATTTTACAGACTGTAAAGTTGTTGATGAATT
 GGTCAAAGTAATGGGGCGGCATAAGCCAGAAAATATCGTTATTGAAATGGCAGCTGAAAATCAGACAACTCAAAAGGGCC
 AGAAAAATTCCGAGAGCGTATGAAACGAATCGAAGAAGGTATCAAAGAATTAGGAAGTCAGATTCTTAAAGAGCATCCT
 GTTGAAGAAATACCTCAATTTGCAAAATGAAAAGCTCTATCTCTATTTATCTCCAAAATGGAAGAGACATGTAATGTGGACCAAGA
 20 ATTAGATATTAATCGTTTAAAGTGATTATGATGTCGATCACATTGTTCCACAAAGTTTCTTAAAGACGATTCAATAGACA
 ATAAGGTCTTAAACGCGTTCTGATAAAAATCGTGGTAAATCGGATAACGTTCCAAGTGAAGAAGTAGTCAAAAAGATGAAA
 AACTATTGGAGACAACCTCTAAACGCCAAGTTAATCACTCAACGTAAGTTTGATAATTTAACGAAAGCTGAACGTGGAGG
 TTTGAGTGAACCTTGATAAAGCTGGTTTATCAACGCCAATTTGGTTGAAACTCGCCAAATCACTAAGCATGTGGCACAAA
 TTTTGGATAGTCGATGAATACTAAATACGATGAAAATGATAAACTTATTCGAGAGGTTAAAGTGATTACCTTAAATCT
 25 AAATTAGTTTCTGACTTCCGAAAAGATTTCGAATTCATAAAGTACGTGAGATTAAACAATTACCATGCCCCATGATGC
 GTATCTAAATCCCGCTTGGAACTGCTTTGATTAGAAATTTCCAAAACCTGAATCGGAGTTTGTCTATGGTGATATATA
 AAGTTTATGATGTTCTGTAAGTCTGAGCAAGAAATAGGCAAGCAACCGCAAAATATTTCTTTTACTCT
 AATATCATGAACCTCTTCAAAACAGAAATTACACTTGCAAAATGGAGAGATTTCGCAACGCCCTCTAATCGAACTAATGG
 GGAAACTGGAGAAATTTGTCTGGGATAAAGGGCGAGATTTTGGCCAGTGCAGCAAGTATTTGTCCATGCCCAAGTCAATA
 30 TTGTCAAGAAAACAGAAGTACAGACAGCGGATTTCTCCAAGGAGTCAATTTTACCAGAAAGAAATTCGGACAAGCTTAT
 GCTCGTAAAAAGACTGGGATCCAAAAAATATGGTGGTTTTGATAGTCCAACGGTAGCTTATTCAGTCTAGTGGTTGC
 TAAGGTGAAAAAGGGAATCGAAGAAGTTAAATCCGTTAAAGAGTTACTAGGGATCACAATTATGGAAGAAGTTCTCT
 TTGAAAAAATCCGATTCGACTTTTGAAGCTTAAAGGATATAAGGAAGTTAAAAAGACTTAATCATTAACCTACCTAAA
 TATAGTCTTTTTGAGTTAGAAAACGGTCGTAAACGGATGCTGGCTAGTGCAGGAGAATTACAAAAAGGAAATGAGCTGGC
 35 TCTGCCAAGCAAAATATGTGAATTTTTTATATTTAGCTAGTCAATTATGAAAAGTTGAAGGGTAGTCCAGAAGATAACGAAC
 AAAACAATTTGTTTGTGGAGCAGCATAAGCATTATTTAGATGAGATTATTGAGCAATCAGTGAATTTTCTAAGCGTGT
 ATTTTAGCAGATGCCAATTTAGATAAAGTTCTTAGTGATATTAACAAACATAGAGACAAACCAATACGTGAACAGCAGA
 AAATATTATTCAATTTATTTACGTTGACGAATCTTGGAGCTCCCGCTGCTTTTAAATATTTTGATACAACAAATGATCGTA
 AACGATATACGTCTACAAAAGAAGTTTAGATGCCACTCTTATCCATCAATCCATCACTGGTCTTTATGAAACACGCATT
 GATTTGAGTCAGCTAGGAGGTGACTGA

-10 promoter (SEQ ID NO: 127)

40 AGTATATTTTAGATGAAGATTATTTCTTAATACTAAATAATGGTATAATACTCTT AATAAATGCAGT

Leader sequence (SEQ ID NO: 128)

AATACAGGGGCTTTTCAAGACTGAAGTCTAGCTGAGACAAATAGTGGATTACGAAATTTTGTAGACAAAAATAGTCTAC

	Direct repeat	Spacer	Direct
45	GAGGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAACT	GAGACCAGTCTCGGAAGCTCAAAGGTCTCGTTTTAGAGCT	
		BsaI	BsaI
	repeat	Spacer	Direct repeat
50	ATGCTGTTTTGAATGGTCCCAAACTTCAGCACACTGAGACTTGTGAGTTCCATGTTTTAGAGCTATGCTGTTTTGAAT		
	GGACTCCATTCAACATTTGCCGATGATAACTTGAGAAAGAGGGTTAATACCAGCAGTCGGATACCTTCCTATTCTTTCTGT		
55	TAAAGCGTTTTCATGTTATAATAGGCAAAAGAAGAGTAGTGTGATCGTCCATTCGACAGCATCGCCAGTCACTATGGCG		
	TGCTGCTAGCGCTATATGCGTTGATGCAATTTCTATGCGCACCCGTTCTCGGAGCACTGTCCGACCGCTTTGGCCGCGCG		
	CCAGTCCGTGCTCGCTTCTGCTACTTGGAGCCACTATCGACTACGCGATCATGGCGACCACACCCGTCCTGTGGATCCTCTA		
	CGCCGGACGCATCGTGGCCGGCATCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGG		
	AAGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGCCGGGGGACTG		
60	TTGGGCGCCATCTCCTTGCATGCACCATTCCTTGGCGGCGGCTGCTCAACGGCCTCAACCTACTACTGGGCTGCTTCCT		
	AATGCAAGAGTCGATAGGGGAGAGCGTCCGACGATGCCCTTGAGAGCTTCAACCCAGTCAGTCTCCTCCGGTGGGCGC		
	GGGGCATGACTATCGTCCGCGCACTTATGACTGTCTTTATCATGCAACTCGTAGGACAGGTGCCGCGAGCGCTCTGG		
	GTCATTTTCGGCGAGGACCGCTTTCGCTGGAGCGCAGCATGATCGGCTGTGCTTGGGATATTCGGAATCTTGCACGC		
	CCTCGCTCAAGCTTCTGTCAGTGGTCCCGCCACCAAGGTTTCGGCGAGAAGCAGGCCATTATCGCCGCGATCTGGCCGCG		
	ACGCGCTGGGCTACGTTTGTGCGGTTTCGCGACGCGAGGCTGGATGGCTTCCCCATTATGATTTCTCTCGCTTCCGCG		
	GGCATCGGGATGCCCGGTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCAAGGATCGCT		
	CGCGGCTCTTACCAGCCTAACTTCGATCATTTGACCGCTGATCGTCACGGCGATTTATGCCGCTTCGGCGAGCACATGGA		

ACGGGTTGGCATGGATTGTAGGCGCCGCCCTATACCTTGTCTGCCTCCCCGCGTTGCGTTCGCGGTGCATGGAGCCGGGGCC
 ACCTCGACCTGAATGGAAGCCGGCGGCACCTCGCTAACGGATTCACCACTCCAAGAATTGGAGCCAATCAATCTTTCGGG
 AGAAGTGTGAATGCGCAAAACCAACCTTGGCAGAACATATCCATCGCGTCCGCCATCTCCAGCAGCCGCACGGCGCAT
 CTCGGGCAGCGTTGGGTCTTGCCACGGGTGCGCATGATCGTGTCTCTGTGTTGAGGACCCGGCTAGGCTGGCGGGGTT
 5 GCCTTACTGGTTAGCAGAATGAATCACCGATACGCGAGCGAAGCTGAAGCGACTGCTGTGCAAAACGTTCTGCCACCTGA
 GCAACAACATGAATGGTCTTCGGTTTTCGTAAAGTCTGGAACCGGAAAGTCCCCTACGTGTGTGAAAGTTG
 CCCGCAACAGAGAGTGGAAACCAACCGGTGATACCACGATACTATGACTGAGAGTCAACGCCATGAGCGGCCCTATTTCTT
 ATTCTGAGTTACAACAGTCCGCACCGTGTCCGGTAGCTCCTTCCGGTGGGCGCGGGGCATGACTATCGTTCGCGCAGCTT
 10 ATGACTGTCTTTTATCATGCAACTCGTAGGACAGGTGCCGCGAGCGCCCAACAGTCCCCCGGCCACGGGGCTGCCAC
 CATACCACGCCGAAACAAGCGCCCTGCACCATTATGTTCCGGATCTGCATCGCAGGATGTGTGGTACCCTGTGGAA
 CACCTACATCTGTATTAACGAAGCGCTAACCGTTTTTATCAGGCTCTGGGAGGCAGAATAAATGATCATATCGTCAATTA
 TTACTCCACGGGAGAGCCTGAGCAAACTGGCTCAGGCATTTGAGAAGCACACGGTCACACTGCTTCCGGTAGTCAAT
 AAACCGGTAAACCAGCAATAGACATAAGCGGCTATTTAACGACCTGCCCTGAACCGACGACCGGGTCAATTTGCTTTC
 15 GAATTTCTGCCATTATCCGCTTATTATCACTTATTCAGGCGTAGCACCAGGCGTTAAGGGCACCAATAAAGTCCCTTAA
 AAAAATTACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATCACA
 GACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCCGCTTGCCTATAATATTTGCCCATGGTGAACACGG
 GGGCGAAGAAGTTGTCCATATTTGGCCACGTTTAAATCAAACTGGTGAACCTCACCCAGGGATTGGCTGAGACGAAAAAC
 ATATTCTCAATAAACCTTTAGGGAAATAGGCCAGGTTTTACCCTAACACGCCACATCTTGCGAATATATGTGTAGAAA
 20 CTGCCGGAATCGTGTGTTTCACTCCAGAGCGATGAAAACGTTTCAGTTTGTCTCATGGAACCGGTGTAACAAGGGT
 GAACACTATCCCATATCACAGCTCACCGTCTTTCATTGGCCATACG

The reverse complement of tracrRNA is in lowercase, Cas9 coding sequence (SEQ ID NO: 126) is boxed and direct repeat sequence in CRISPR is in bold. Promoter sequences were
 25 predicted by neural network algorithm (http://www.fruitfly.org/seq_tools/promoter.html). The
 two unique sites, *Sa*I (GTCGAC) and *Xba*I (TCTAGA) highlighted in bold italicised black
 are utilised to isolate the CRISPR/Cas9 construct for cloning into M13mp18. The
 pACYC184 backbone sequence is italicised.

30 The information of Appendix 1 above is alternatively presented as follows.

pCas9 plasmid sequence (SEQ ID NO: 125)

Cas 9 gene, CRISPR expression locus and tracrRNA (all from *S. pyogenes*)

35 GAATTCGGATGAGCATTATCAGGCGGGCAAGAATGTGAATAAAGCGGATAAACTGTGCTTATTTTCTTTACGG
 TCTTTAAAGGCCGTAATATCCAGCTGAACGGTCTGTTTATAGGTACATTGAGCAACTGACTGAAATGCCCTCAAAATGT
 TCTTTACGATGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTCTCCATTTTAGCTTCTTAGCTCCTGA
 40 AAATCTCGATAACTCAAAAATACGCCGGTAGTGATCTTATTTCATTATGGTGAAGTTGGAACTCTTACGTGCCGAT
 CAACGTCTCATTTTCGCCAAAAGTTGGCCACGGGCTTCCCGGTATCAACAGGGACACCAGGATTTATTTATTCTGCGAAG
 TGATCTTCCGTCACAGGATTTATTCGGCGCAAGTGCCTCGGGTGATGCTGCCAACTTACTGATTTAGTGATGATGGT
 45 GTTTTTGAGGTGCTCCAGTGGCTTCTGTTTCTATCAGCTGTCCCTCCTGTTTCACTGACTGACGGGGTGGTGGTAACGGC
 AAAAGCACCCCGGACATCAGCGCTAGCGGAGTGATATCTGGCTTACTATGTTGGCACTGATGAGGGTGTGAGTGAAGTG
 CTTATGTTGGCAGGAGAAAAAGGCTGCACCGGTGCGTCAGCAGAAATATGTGATACAGGATATATCCCGCTTCTCGCTC
 50 ACTGACTCGCTACGCTCGGTGCTTCGACTGCGGCGAGCGGAAATGGCTTACGAACGGGGCGGAGATTTCTTGAAGATGC
 CAGGAAGATACCTAACAGGGAAGTGAGAGGGCCGCGCAAGCCGTTTTTCCATAGGCTCCGCCCCCTGACAAGCATCA
 CGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGCGGCTCCC
 TCGTGCGCTCTCTGTTCTCTGCTTTTCGGTTTACCGGTGTCTTCCGCTGTTATGGCCGCGTTTGTCTCATTCCACGCCCT
 55 GACACTCAGTTCCGGTAGGCAAGTTCGCTCCAAGCTGGACTGTATGCACGAACCCCGCTTCACTCCGACCGCTGCGCCT
 TATCCGGTAACATATCGTCTTGAGTCCAACCCGGAAGACATGCAAAAGCACCAGTGGCAGCAGCCACTGGTAATTGATTT
 AGAGGAGTTAGTCTTGAAGTCATGCGCGGTTAAGGCTAACTGAAAGGACAAAGTTTGGTGACTGCGCTCCCTCCAAGCC
 60 AGTTACCTCGGTTCAAAGAGTTGGTAGCTCAGAGAACCCTCGAAAAACCGCCTGCAAGGCGGTTTTTCGTTTTAGAG
 CAAGAGATTACGCGCAGACCAAAACGATCTCAAGAAGATCATCTTATTAATCAGATAAAATATTTCTAGATTTTCAGTGCA
 ATTTATCTCTTCAAAATGAGCACTGAAGTCAGCCCCATACGATATAAGTTGTAATTTCTCATGTTTGACAGCTTATCATC
 GATAAGCTTTAATGCGGTAGTTTATCAGAGTTAAATTTGCTAACGCAAGTCAAGCAGGCTGATGAAATCAACAATGCGCT
 65 CATCGTATCTTCGGCACCGTCACCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCTCTTGGCGG
 ATTACGAATCATCTGTGGAGCTTAGTAGGTTAGCAAGATGGCAGCGCCTAAATGTAGAATGATAAAAGGATTAAAGAG
 ATTAATTTCCCTAAAAATGATAAAACAAGCGTTTTGAAAGCGCTTGTTTTTTGGTTTGCAGTCAGAGTAGAATAGAAGT
 ATCAAAAAAGCAGCCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTATTTTAAGTTGCTATGCTGTTTTG
 70 AATGGTTCCCAACAAGATTATtttataaacTttataacaaataatcaaggagaaattcaagaaatttatCAGCCATAAAA
 CAATACTTAATACTATAGAATGATAACAAAATAAATACTTTTTTAAAGAAATTTTGTGTTATAATCTATTATTATTAAG
 TATtggttaatatTTTTgaagagatatTTTgaaaaagaaaaTtaaaagcataTTAACTAATTTCCGAGGTCATTAATA
 CTATTATTGAATCATCAAACTCATTATGGATTTAATTTAACTTTTTTATTTTAGGAGGCAAAAATGGATAAGAAATACT

CAATAGGCTTAGATATCGGCACAAATAGCGTCGGATGGGCGGTGATCACTGATGAATATAAGGTTCCGTCTAAAAAGTTCA
 AAGGTTCTGGGAATAATACAGCCGCCACAGTATCAAAAAAATCTTATAGGGGCTCTTTATTATTGACAGTGGAGAGACAGC
 GGAAGCGACTCGTCTCAAACGGACAGCTCGTAGAAGGTATACACGTGGGAAGAATCGTATTTGTTATCTACAGGAGATTTT
 TTTCAAATGAGATGGCGAAAGTAGATGATAGTTTCTTCATCGACTTGAAGAGTCTTTTTTGGTGGGAAGAAGCAAGAAG
 CATGAACGTCATCTCTATTTTGGAAATATAGTAGATGAAGTTGCTTATCATGAGAAATATCCAATCTCTATCATCTCGCG
 AAAAAAATTTGGTAGATTCTACTGATAAAGCGGATTTGCGCTTAATCTATTTGGCCTTAGCGCATATGATTAAGTTTCGTG
 GTCATTTTTTATTGATTGAGGGAGATTTAAATCCTGATAAATAGTATGTTGGACAAACTATTATCCAGTTGGTACAAACCTAC
 AATCAATTATTTGAAGAAAACCTATTAAACGCAAGTGGAGTAGATGCTAAAGCGATTCTTTCTGCACGATTGAGTAAATC
 AAGACGATTAGAAAATCTCATTGCTCAGCTCCCCGGTGAGAAGAAAAATGGCTTATTTGGGAATCTCATTGCTTTGTCAT
 TGGGTTTGACCCCTAATTTTAAATCAAATTTGATTGTTGGCAGAAGATGCTAAATTACAGCTTTCAAAAGATACTTACGAT
 GATGATTTAGATAATTTATTGGCGCAAATGGAGATCAATATGCTGATTGTTTTGGCAGCTAAGAAATTTATCAGATGC
 TATTTTACTTTTATATCTTAAGAGTAAATACTGAAATACTAAGGCTCCCCCTATCAGCTTCAATGATTAAACGCTACG
 ATGAACATCATCAAGACTTGACTCTTTTAAAGCTTTAGTTTCGACAACTTCCAGAAAAGTATAAAGAAATCTTTTTT
 GATCAATCAAAAACGCGATATGCGGTTATATTGATGGGGAGCTAGCCAAAGAAATTTTATAAATTTATCAAAACCAAT
 TTTAGAAAAATGGATGGTACTGAGGAATATTGGTGAACATAAATCGTGAAGATTGCTGCGCAAGCAACGGACCTTTG
 ACAACGGCTCTATTCCCCTCAAATTTCACTTGGGTGAGCTGCATGCTATTTTGAGAAGACAAGAAGACTTTTATCCATTT
 TTAAGAGACAATCGTGAGAAGATTGAAAAATCTTGACTTTTCGAATTCCTTATTATGTTGGTCCATTGGCGCGTGGCA
 TAGTCGTTTTGTCATGGATGACTCGGAAGTCTGAAGAAACAATTAACCCATGGAATTTTGAAGAAGTTGTCGATAAAGGTG
 CTTGAGCTCAATCATTATTGAACGCATGACAACTTTGATAAAAAATCTTCAAATGAAAAAGTACTACCAAAACATAGT
 TTGCTTTATGCGATTATTGCTCAAAATATTAAGATAAAGATTTTTTGGATAATGAAGAAATGAAGATATCTTAGAGATAT
 TGTTTTAAACATTGACCTTATTGAAGATAGGGAGATGATTGAGGAAGACTTAAACATATGCTCACCTCTTTGATGATA
 AGGTGATGAAACAGCTTAAACGTCGCCGTTATACCTGGTGGGGACGTTTGTCTCGAAAATGATTAAATGGTATTAGGGAT
 AAGCAATCTGGCAAAACAAATATTAGATTTTTTGAATCAGATGGTTTTGCCAATCGCAATTTTATGACGCTGATCATGA
 TGATAGTTTTCATTTAAAGAGACATTCAAAAGCACAAGTGTCTGGACAAGGCGATAGTTTACATGAACATATTGCAA
 ATTTAGCTGGTAGCCCTGCTATTAAAAAGGTATTTTACAGACTGTAAGAGTTGTTGATGAATGGTCAAAGTAATGGGG
 CGGCATAGCCAGCAAAAAATCTGTTTGAATGGACGCTGAAAAATCAGACAACCTCAAAAGGGCCGAAAAAATTCGCGGAG
 GCGTATGAAACGAATCGTAGAAGGTATCAAGAATTAGGAAGTCAGATTCTTAAAGAGCATCCTGTTGAAAAATCTCAAT
 TGCAAAATGAAAGCTCTATCTTATTATCTCCAAAATGGAAGAGACATGTATGTTGACCAAGAATTAGATATTAAATCGT
 TTAAGCACTGGTTTTATCAACCGCCAAATTTGTTGAACTCGCCAAATCACTAAGCATGTGGCACAATTTTGGATAGTCGCAT
 TTCTGATAAAAAATCGTGGTAATCGGATAACGTTCCAAGTGAAGAGTAGTCAAAAGATGAAAACTATTGGAGACAAC
 TTCTAAACGCCAAGTTAATCACTCAACGTAAGTTTGATAATTTAACGAAAGCTGAACGTGGAGGTTTGAAGTGAACCTTGAT
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 TCCGAAAAGATTTCAATTTCTATAAAGTACGTGAGATTAACAATTAACCATCATGCCATGATGCGTATCTAAATGCCGTC
 GTTGGAACTGGTTGATTAAAGAAATATCCAAAATGAACTCGCCAAATCACTAAGCATGTGGCACAATTTTGGATAGTCGCAT
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 TCAAAACGAAATTAACATTGCAAAATGGAGAGATTGCGCAACGCCCTCTAATCGAACTAATGGGAAACTGGAGAAATTT
 GTCTGGGATTAAGGGCGCAATTTGCCACAGTCGCAAAAGTATTGTCCATGCCCAAAGTCAATATTGTCAAGAAACAGAG
 AGTACAGACAGGCGGATTCTCCAAGGAGTCAATTTTACCAAAAGAAATTCGGACAAGCTTATTGCTCGTAAAAAGACT
 GGGATCCAAAAAATATGGTGGTTTTGATAGTCCAACGGTAGCTTATTCACTCCTAGTGGTGGTGAAGGTGGAAAAAGGG
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 TAGAAAACGGCTCGTAAACGGGCTGCTGGCTAGTCCGCGAAGTACAAAAAGGAAATGAGCTGGCTCGCCAAAGCAATAT
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 GGAGCAGCATAGCATATTATTAGATGAGATTATTGAGCAATCAGTGAATTTTCTAAGCGTGTATTATTAGCAGATGGCA
 ATTTAGATAAAGTCTTAGTGCATATAACAAACATAGAGACAAACCAATACGTGAACAGCAGAAAAATATTATTCAATTTA
 TTTACGTTGACGAATCTTGGAGCTCCCGCTGCTTTTAAATATTTTGATACAACAATGATCGTAAACGATATACGCTCTAC
 AAAAGAAAGTTTATAGATGCCACTCTTATCCATCAATCCATCACTGGTCTTTATGAAACACGCAATTGATTGAGTCAGCTG
 GAGTGATCTGAAGTATATTtagatgaagattatttctaataactaaaaatatggtTataaactctTATAATAATCGCAGT
 AATACAGGGGCTTTTCAAGACTGAAGTCTAGCTGAGACAAATAGTCCGATTACGAAATTTTTTATAGACAAAAATAGTCTAC
 GAGTGTATTAGAGCTATGCTGTTTTGAATGGTCCCAAACTGAGACCAAGTCTCGGAAGCTCAAAAGGCTCGTTTTAGAGCT
 ATGCTGTTTTGAATGGTCCGAAACTTCAGCACATGAGACTTGTGAGTTCCATGTTTTAGAGCTATGCTGTTTTGAAT
 GGACTCCATTCAACATTGCGGATGATAACTTGAGAAAGAGGGTTAATACCAGCAGTCGGATACCTTCTTATCTTTCTGT
 TAAAGCTTTTATGATTTAATAGGCAAAAGAGAGTAGTGTGATCGTCCATTCCGACAGCATCGCCAGTCACTATGGCG
 TGCTGCTAGCGCTATA TGCGTTGATGCAATTTCTATGCGCACCCGTTCTCGGAGCACTGTCCGACCGCTTTGGCGCGCCG
 CCAGTCTGCTCGCTTCGCTACTTTGGAGCCACTATCGACTACGCGATCATGGCGACCACACCCGCTCTGTGGATCCTCTA
 CGCCGGAGCGATCGTGGCGCGCATCACCGGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCAGTGGGG
 AAGATCGGGCTCGCAATCTGGGCTCATGAGCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCCCGCTGGCGCGGGGACTG
 TTGGCGCCATCTCCTTGATGACCACTTCTTTCGGCGCGCGGTGCTCAACGGCTCAACCTACTACTGGGCTGCTTCCT
 AATGCGAGGATCGCATAGGGGAGAGCGTCGACCGATGCCCTTGAGAGCCTTCAACCCAGTCAAGCTCCTTCCGGTGGGCGC
 GGGGACTGACTATCTGTCGCGCACTTATGACTGTCTTTCTTATCATGCAACTCGTAGGACAGGTGCGCGCAGCGCTCTGG
 GTCATTTTTCGGCGAGGACCGCTTTCGCTGGAGCGCGACGATGATCGGCTGTCGCTTGGGATTCGGAATCTTGACGCG
 CCTCGCTCAAGCCTTCGCTACTGGTCCCGCCACCAACGTTTCGGCGAGAAGCAGGCCATTATCGCCGCGATGGCGCGC
 ACGCGCTGGGCTAGCTCTTGGTGGGCTTCGCGACGCGAGGCTGGATGGCCTTCCCATATTGATTCTTCTCGCTTCCGGT
 GGCATCGGGATGCCGCGTTGCGAGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCAAGGATCGCT
 CGCGGCTCTTACCAGCCTAACTTCGATCATTGGACCGCTGATCGTCACGGCGATTATGCCGCTCGCGGAGCAGATGGA
 ACGGGTTGGCATGGATTGTAGGCGCGCCCTATACCTTGTCTGCCCTCCCGCGTTGCGTCCGCGGTGCATGGAGCCGGGCC
 ACCTCGACCTGAATGGAAGCCGGCGGCACCTCGCTAACGGATTCAACACTCCAAGAATTGGAGCCAAATCAATCTTTGCGG
 AGAAGTGTGAATGCGCAAAACCAACCTTGGCAGAACATATCCATCGCGTCCGCCATCTCCAGCAGCCGACCGCGCGCAT
 CTCGGGACGGTGGGTCTCGGCCACGGGTGCGCATGATCGTGCTCTGTGCTTGGAGACCCGGCTAGGCTGGCGGGGT
 GCCTTACTGGTTAGCAGAAATGAATCACCAGTACGCGAGCGAAGCTGAAGCGACTGCTGCTGCAAAACGCTGCGACCTGA
 GCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTAAGTCTGGAACCGCGGAAGTCCCCCTACGTGCTGCTGAAGTTG
 CCCGACAGAGAGTGGAAACCAACCGGTGATACACGATACTGACTGAGAGTCAACGCCATGAGCGGCCCTCAATTTCTT
 ATTCGTAGTTTACAACAGTCCGACCGCTGTCCGGTAGCTCCTTCCGGTGGGCGCGGGGATGACTATCGTCCGCGCACT
 ATGACTGCTCTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCCCAACAGTCCCCCGGCCACGGGCGCTGCCAC
 CATCCCGCCGAAACCAAGCCCGCTGACCAATTTATTCGGATCTGCATCGCAGGATGCTGCTGGCTACCTGTGGAA
 CACCTACATCTGTATTAAACAGCGCTAACCGTTTTTATCAGGCTCTGGGAGGCAGAAATAATGATCATATCGTCAATTA
 TTACCTCCACGGGAGAGCCTGAGCAAACTGGCCTCAGGCATTTGAGAAGCACAGGTCACTGCTTCCGGTAGTCAAT
 AAACCGGTAAACGCAATAGACATAAGCGGCTATTTAACGACCTTGCCCTGAACCGACGACCGGGTCAATTTGGCTTC
 GAATTTCTGCCATTATCCGCTTATTATCACTTATTCAGGCTAGACACGAGCGTTTAAAGGCACCAATAACTGCCTTAA

5 AAAAAATTACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATCACA
 GACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAATATTGCCCATGGTGAAAACGG
 GGGCGAAGAAGTTGTCCATATTGGCCACGTTTAAATCAAACTGGTGAAACTACCCAGGGATTGGCTGAGACGAAAAAC
 ATATTCTCAATAAACCCCTTAGGGAAATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAA
 CTGCCGGAATCGTCGTGGTATTCCTCCAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAACGGTGAACAAGGGT
 GAACACTATCCCATATCACCAGCTCACCGTCTTTCATTGCCATACG

Backbone vector pACYC184 sequence is italicised, sequence positions are numbered from
 10 G of *EcoRI* site underlined. The reverse complement of tracrRNA is in italicised bold
 located from 1844 to 1929, Cas9 initiation and termination codons are indicated by bold
 three letters, starting at nucleotide No. 2225 and ending at 6331 followed by leader
 sequence 6389-6483 indicated by italicised bold letters, first, second and third direct repeat
 sequences are underlined, between the first and second direct repeat in which spacer
 15 cloning region is located.

This spacer cloning region contains two inverted *BsaI* sites indicated by bold italicised
 letters 5'-GAGACC-3' and 5'-GGTCTC-3' for creating 5' four bases protruding spacer
 cloning sites 5'-GTTT-3' and 5'-TTTT-3', respectively. Promoter sequences were predicted
 20 as above, indicated by lower case for forward promoter for Cas9 and leader sequence and
 italicised lower case for reverse promoter for tracrRNA, the putative transcription start site
 is indicated by bold uppercase. The two unique sites, *SaI* (GTCGAC) and *XbaI*
 (TCTAGA) highlighted in bold italicised black are utilised to isolate the CRISPR/Cas9
 construct for cloning into M13mp18.
 25

Appendix 2

Modification of spacer cloning site on pCas9

This system exploits the fact that expression of a minigene yields a certain oligopeptide
 30 that depletes the tRNA pool in the host cell, when induced for expression, which leads to
 the disruption of protein synthesis (Tenson T, Vega-Herrera J, Kloss P, Guameros G,
 Mankin AS. J. Bacteriol. 1999; 181:1617-1622). Applying this concept to cloning the
 spacer sequence: when the spacer sequence is inserted this interrupts the minigene and
 the oligopeptide expression does not occur, thus tRNA is not depleted, thus only the
 35 bacteria harbouring a plasmid with insert can survive under conditions of induced
 expression; whilst bacteria harbouring the plasmid without insert cannot grow. The
 following (SEQ ID NO: 129) is the sequence structure. In order to construct this structure,
 bacteria should express the *lacI* repressor constitutively to switch off minigene
 transcription. Recombinant selection should be performed in the presence of IPTG to
 40 induce transcription.

BsaI recognition sites are underlined. lac operator 3 and 1 are bold italicised. Tac promoter is in bold letters, -35 and -10 region in the promoter sequence are underlined, Shine-Dalgarno sequence is boxed, first dipeptide is in bold M and R followed by three consecutive termination codons italicised TAA. Tryptophan terminator signal sequence is employed, indicated by italicised letters.

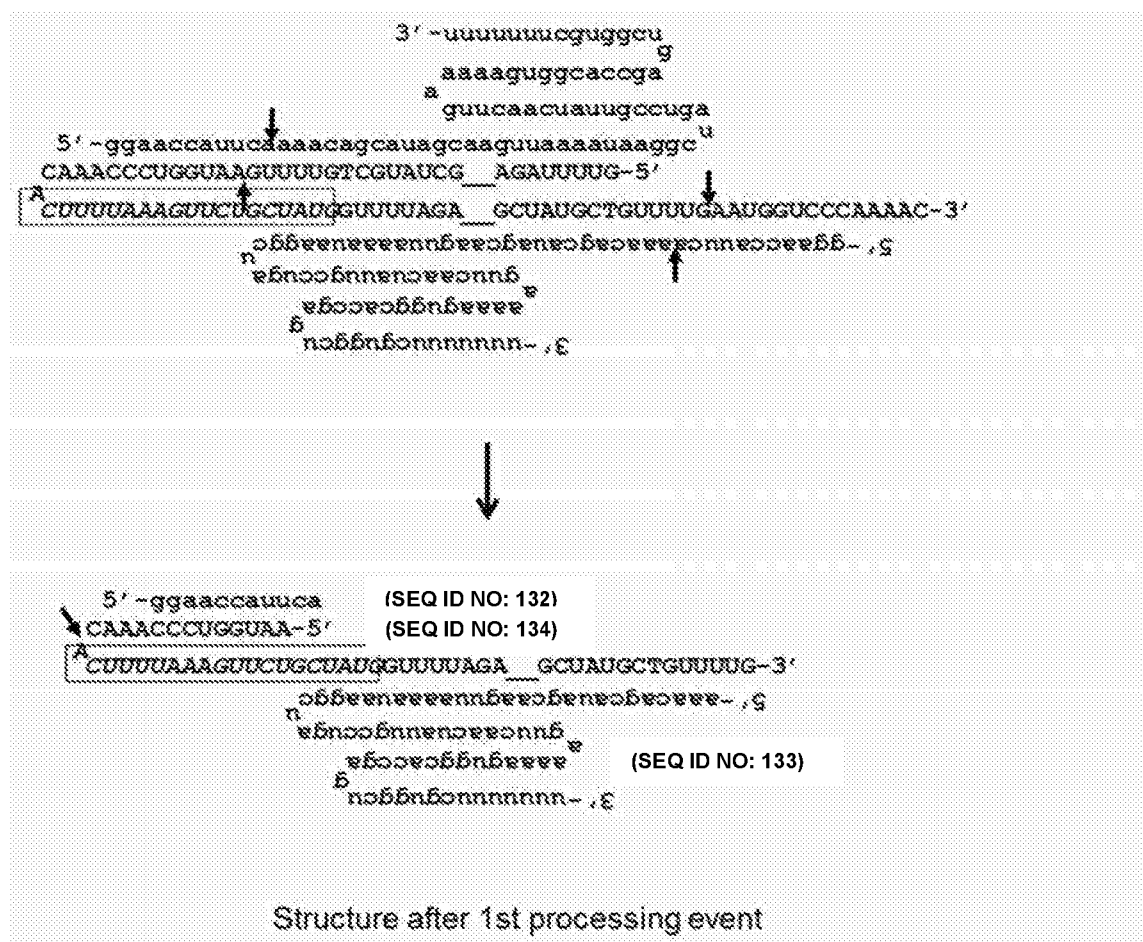
Structure after 1st processing event

(SEQ ID NO: 132)

```
5' - ggaacccauuca  
CAAACCUGGUAA  
pre-crRNA      a      cuuuuaaaguucugcuaugGUUUUAGA GCUAUGCTGUUUUG  
                                     uaaaaugaacgauacgacaaa  
agccacggugaaaaagucaacuauugccugaucggaa  
g      ucggugcuuuuuuu-3'      tracrRNA
```

15 The first processing event may also be depicted as follows: tracrRNAs indicated by lower case letters hybridise to the direct repeat region of pre-crRNA indicated by upper case bold black letters. Bacterial RNase III cleaves the double-stranded RNA region at indicated position with arrows, which is first processing event. Phosphodiester bond between 22nd and 23rd base in the first and second direct repeat are cleaved. Italicised spacer sequence

20 CR90 is boxed.



Second processing event

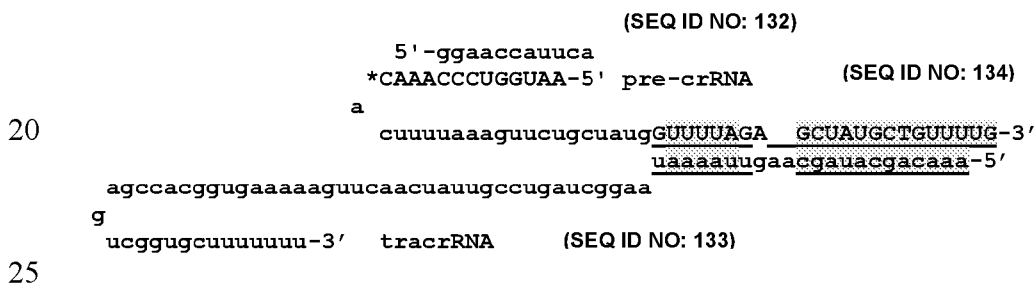
The 2nd cleavage point (*) is around 20 nt away from the 3' end of the spacer sequence.

"Note that the 2nd processing event occurs at a specific distance from the 1st cleavage

- 5 within the repeats. Considering that spacer sequences are not identical among each other, it is thus likely that the 2nd processing event within the spacers is distance-dependent rather than sequence-dependent" (see Supplementary figure 2 legend in Nature. Mar 31, 2011;471 (7340):602-607).

- 10 Although the above-referenced article does not particularly specify the enzyme and/or mechanism involved in this 2nd processing event, it is most likely Cas9 is involved in this 2nd cleavage. Considering this cleavage position within the spacer sequence, the whole spacer sequence is not contributing to the target recognition, instead only 20 nt spacer

15

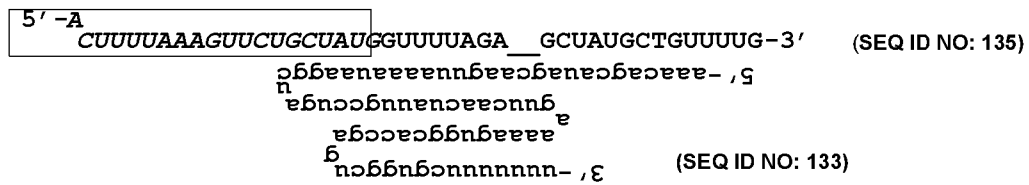


The second processing event may also be depicted as follows: The 2nd cleavage point indicated by arrows around 20 nt away from the 3' end of the spacer sequence in the above figure.

30

The above note quoted from Nature, and our comment, applies.

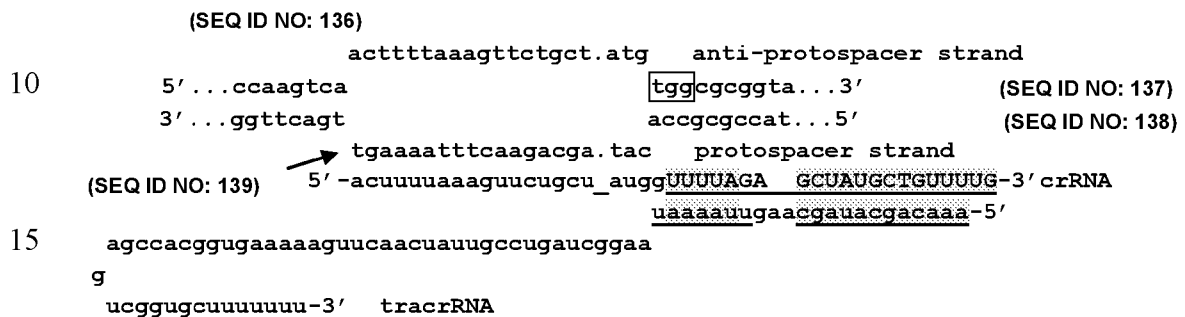
The italicised spacer sequence CR90 is in box.



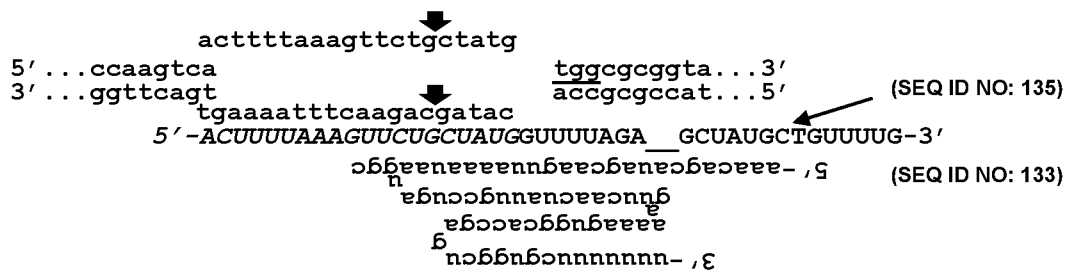
Structure after 2nd processing event

Target DNA cleavage

- 5 The part of the target beta-lactamase DNA sequence containing CR90 is shown. crRNA hybridises to its complementary sequence of the target region, Cas9 cleavage points are indicated by dot “.” and the PAM sequence tgg is indicated in the box.



- 20 An alternative depiction of the target DNA cleavage is shown below. Here, the part of the target beta-lactamase DNA sequence containing CR90 proto-spacer sequence is shown in lower case bold black letters. crRNA hybridises to its complementary sequence of the proto-spacer strand, Cas9 cleavage points are indicated by dot arrows and the PAM
- 25 sequence tgg is under lined.



Annealing to proto-spacer DNA and cleavage points

5 **Example 2**

The following experiments describe some proof-of-concept experiments performed to demonstrate that the CRISPR-Cas9 system can be used to inactivate antibiotic resistance in bacteria. They describe the construction of a generally applicable DNA cassette, described in the Examples to deliver the CRISPR-Cas9 system plus a derivative carrying a spacer sequence targeted against an antibiotic resistance gene for delivery by naked DNA transformation and bacteriophage infection and also to demonstrate inhibition of the spread of antibiotic resistance by plasmid conjugation.

15 **Construction of pNB100**

pNB100 is a vector to express the CRISPR-Cas9 system in *E.coli* with the appropriate unique restriction site, *Bsa* I, to clone any desired spacer sequence between two direct repeats in the CRISPR locus. The backbone of the vector is derived from pACYC184 and the CRISPR-cas9 locus is inserted into *Eco* RV site of the vector. Three regions of the CRISPR-cas9 locus were amplified by PCR from the genomic DNA of *Streptococcus pyogenes* strain SF370, purchased from the ATCC, and assembled by Gibson assembly (Gibson DG, et.al. Nature Methods 2009; 6: 343-345) along with the pACYC184 vector in the reaction. The sequence of the final construct was verified by Sanger sequencing. The CRISPR-Cas9 activity was confirmed using a derivative of pNB100, pNB102, carrying a spacer sequence targeted against the *beta lactamase* genes of the bacterial transposons Tn3 and Tn1.

The amplified sequence of the three regions and amplicon image on the gel

The following sequences are the three regions amplified by PCR. Underlined sequences are template-specific primer sequences, bold letters are overlapping sequences used for Gibson assembly.

5

1. Fragment 1 (SEQ ID NO: 140), tracrRNA-cas9 : amplicon length = 4758 bp

Forward primer is from 854170 to 854193 and reverse primer is from 858867 to 858848 on *S. pyogenes* SF370 genomic DNA.

10 **ATGCCGGTACTGCCGGCCTCTTGCGGGAT**CCAGAAGTCTTTTCTTGCACTGTTTCCTTTCTTTATGATAGTTTACGAAATCATCC
TGTGGAGCTTAGTAGGTTAGCAAGATGGCAGCGCTAAATGTAGAATGATAAAAGGATTAAGAGATTAATTTCCCTAAAAATGATAA
AACAAGCGTTTTGAAAGCGCTTGTTTTTTGGTTTGCAGTCAGAGTAGAATAGAAGTATCAAAAAAGCACCGACTCGGTGCCACTTT
TTCAAGTTGATAACGGACTAGCCTTATTTAACTTGCTATGCTGTTTTGAATGGTTCCAACAAGATTATTTATACTTTTATAACAA
15 ATAATCAAGGAGAAATTCAAAGAAATTATCAGCCATAAAACAATACTTAATACTATAGAATGATAACAAAATAAACTACTTTTAAA
AGAATTTTGTGTATAATCTATTTATTATTAAGTATTGGGTAATATTTTTGAAGAGATATTTGAAAAAGAAAAATTAAGCATATT
AACTAATTTCCGAGGTCATTAATACTATTATGAAATCATCAAACTCATTATGGATTAAATTTAACTTTTATTTTAGGAGGCAAAA
AATGGATAAGAAATCTCAATAGGCTTAGATATCGGCACAAATAGCGTCGGATGGGCGGTGATCACTGATGAATATAAGGTTCCGTCT
20 AAAAAAGTTCAAGGTTCTGGGAAATACAGACCGCCACAGTATCAAAAAAATCTTATAGGGGCTCTTTTATTGACAGTGGAGAGACAG
CGGAAGCGACTCGTCTCAAACGGACAGCTCGTAGAAGGTATACACGTCGGAAGAATCGTATTTGTTATCTACAGGAGATTTTTTCAA
TGAGATGGCGAAAGTAGATGATAGTTTCTTTCATCGACTTGAAGAGTCTTTTTTGGTGAAGAAGACAAGAAGCATGAACGTCATCCT
ATTTTTGGAATATAGTAGATGAAGTTGCTTATCATGAGAAATATCCAATCTATCATCTGCGAAAAAATTTGGTAGATTCTACTG
ATAAAGCGGATTTGCGCTTAATCTATTTGGCCTTAGCGCATATGATTAAAGTTTCGTGGTCATTTTTGATTGAGGGAGATTTAAATCC
TGATAATAGTGATGTGGACAACTATTTATCCAGTTGGTACAAACCTACAATCAATTATTTGAAGAAACCCCTATTAACGCAAGTGGA
25 GTAGATGCTAAAGCGATTCTTCTGCACGATTGAGTAAATCAAGACGATTAGAAAATCTCATTGCTCAGCTCCCCGGTGAGAAGAAAA
ATGGCTTATTTGGGAATCTCATGTGCTTGTCTTGGGTTGACCCCTAATTTTAAATCAAATTTTGATTGGCAGAAGATGCTAAATT
ACAGCTTTCAAAGATACTTACGATGATGATTAGATAATTTATTGGCGCAAATGGAGATCAATATGCTGATTGTTTTTGGCAGCT
AAGAATTTATCAGATGCTATTTTACTTTCAGATATCCTAAGAGTAAATACTGAAATAACTAAGGCTCCCTATCAGCTTCAATGATTA
AACGCTACGATGAACATCATCAAGACTTGACTCTTTTAAAGCTTTAGTTTCGACAACAACCTCCAGAAAAGTATAAAGAAATCTTTT
TGATCAATCAAAAAACGGATATGCAGGTTATATTGATGGGGAGCTAGCCAAGAAGATTTTATAAATTTATCAAAACCAATTTTAGAA
30 AAAATGGATGGTACTGAGGAATATTGGTGAAACTAAATCGTGAAGATTGTCTGCGCAAGCAACGGACCTTTGACAACGGCTCTATTC
CCCATCAAATTCACCTTGGGTGAGCTGCATGCTATTTTGAGAAGACAAGAAGACTTTTATCCATTTTAAAGACAATCGTGAGAAGAT
TGAAAAATCTTGACTTTTCGAATTCCTTATTATGTTGGTCCATTGGCGCGTGGCAATAGTCGTTTTGCATGGATGACTCGGAAGTCT
GAAGAACAAATTTACCCATGGAAATTTGAAGAAGTTGTGCGATGAAGGTGCTTCAGCTCAATCATTTTGAACCGCATGACAACTTTG
ATAAAAAATCTTCCAAATGAAAAAGTACTACCAAAACATAGTTTGCTTTATGAGTATTTACGGTTTATAACGAATTGACAAAGGTCAA
35 ATATGTTACTGAAGGAATGCGAAAACAGCATTCTTTCAGGTGAACAGAAGAAAGCCATTGTTGATTTACTCTTCAAAACAAATCGA
AAAGTAACCGTTAAGCAATTAAGAAGATTATTTCAAAAAATAGAATGTTTTGATAGTGTTGAAATTCAGGAGTTGAAGATAGAT
TTAATGCTTCATTAGGTACCTACCATGATTTGCTAAAAATTATTAAGATAAAGATTTTGGGATAATGAAGAAAAATGAAGATATCTT
AGAGGATATTGTTTTACATTGACCTTATTTGAAGATAGGGAGATGATTGAGGAAAGACTTAAACATATGCTCACCTCTTTGATGAT
AAGGTGATGAAACAGCTTAAACGTCGCCGTTTACTGGTTGGGGACGTTTGTCTCGAAAATTGATTAATGGTATTAGGGATAAGCAAT
40 CTGGCAAAACAAATATTAGATTTTGAATCAGATGGTTTGGCAATCGCAATTTTATGCAGCTGATCCATGATGATAGTTTGACATT
TAAAGAAGACATTCAAAAAGCACAAGTGTCTGGACAAGGCGATAGTTTACATGAACATATTGCAAATTTAGCTGGTAGCCCTGCTATT
AAAAAAGGTATTTACAGACTGTAAGATTGTTGATGAATTGGTCAAAGTAATGGGGCGGCATAAGCCAGAAAATATCGTTATTGAAA
TGGCACGTGAAAATCAGACAACCTCAAAAGGGCCAGAAAATTCGCGAGAGCGTATGAAACGAATCGAAGAAGGTATCAAGAATTAGG
AAGTCAGATTCTTAAAGAGCATCCTGTGAAAATACTCAATTGCAAAATGAAAAGCTCTATCTCTATTATCTCCAAAATGGAAGAGAC
45 ATGTATGTGGACCAAGAATTAGATATTAATCGTTTAAAGTGATTATGATGTGATCAGATCATTGTTCCACAAAGTTTCTTAAAGACGATT
CAATAGACAATAAGGCTTAAACGCGTTCTGATAAAAAATCGTGGTAAATCGGATAACGTTCCAAGTGAAGAAGTAGTCAAAAAGATGAA
AACTATTGGAGACAACCTTCTAAACGCCAAGTTAATCACTCAACGTAAGTTTGATAATTTAACGAAAGCTGAACGTGGAGGTTTGAGT
GAACCTTGATAAAGCTGGTTTTATCAAAACGCCAATTGGTTGAAACTCGCCAAATCACTAAGCATGTGGCACAATTTTGGATAGTCGCA
TGAATACTAAATACGATGAAAAATGATAAACTTATTCGAGAGGTTAAAGTGATTACCTTAAATCTAAATTAGTTTCTGACTTCCGAAA
50 AGATTTCCAATTCTATAAAGTACGTGAGATTAACAATTACCATCATGCCATGATGCGTATCTAAATGCCGTCGTTGGAAGTCTGTTG
ATTAAGAAATATCCAAAACCTTGAATCGGAGTTGTCTATGGTGATTATAAAGTTTATGATGTTGCTGATAAATGATTGCTAAGTCTGAGC
AAGAAATAGGCAAGCAACCGCAAAATATTTCTTTTACTCTAATATCATGAACCTTCTTCAAAACAGAAATTACACTTGCAATGGAGA
GATTTCGCAACGCCCTCTAATCGAAACTAATGGGGAACTGGAGAAATGTCTGGGATAAAGGGCGAGATTTTGCCACAGTGCGCAAA
GTATTGTCCATGCCCAAGTCAATATTGTCAAGAAAACAGAACTACAGACAGGCGGATTCTCCAAGGAGTCAATTTTACCAAAAAGAA
55 ATTCGGACAAGCTTATTGCTCGTAAAAAGACTGGGATCCAAAAAATATGGTGGTTTGATAGTCCACAGGTAGCTTATTCAGTCTCT
AGTGGTTGCTAAGGTGAAAAAGGGAATCGAAGAAGTTAAATCCGTTAAAGAGTTTACTAGGGATCACAATTTATGGAAGAAGTTCC
TTTGAAAAAATCCGATTGACTTTTTTGAAGCTAAAGGATATAAGGAAGTTAAAAAGACTTAATCATTAACTACCTAAATATAGTC

TTTTTGAGTTAGAAAACGGTCGTAAACGGATGCTGGCTAGTGCCGAGAATTACAAAAAGGAAATGAGCTGGCTCTGCCAAGCAAATA
TGTGAATTTTTTATATTTAGCTAGTCATTATGAAAAGTTGAAGGGTAGTCCAGAAGATAACGAACAAAAACAATTGTTTGTGGAGCAG
CATAAGCATTATTTAGATGAGATTATTGAGCAAAATCAGTGAATTTCTAAGCGTGTTATTTAGCAGATGCCAATTTAGATAAAGTTC
TTAGTGCATATAACAAACATAGAGACAAACCAATACGTGAACAAGCAGAAAAATTATTCATTATTTACGTTGACGAATCTTGGAGC
5 TCCCGCTGCTTTTAAATATTTTGATACAACAATTGATCGTAAACGATATACGCTACAAAAGAAGTTTATAGATGCCACTCTTATCCAT
CAATCCATCACTGGTCTTTATGAAACACGCATTGATTTGAGTCAGCTAGGAGGTGACTGATGGC**CACGTGA**ACTATATGATTTTCCGC
AGTATA

2. Fragment 2 (SEQ ID NO: 141), Leader and first direct repeat : amplicon length = 276 bp
10 Forward primer is from 860648 to 860671 and reverse primer is from 860862 to 860806 on
S. pyogenes genomic DNA.

ATTGATTTGAGTCAGCTAGGAGGTGACTGATGGCCACGTGA**ACTATATGATTTTCCGC**AGTATATTTTAGATGAAGATTATTTCTTAA
TAACTAAAAATATGGTATAATACTCTTAATAAATGCAGTAATACAGGGGCTTTTCAAGACTGAAGTCTAGCTGAGACAAATAGTGCGA
15 TTACGAAATTTT**TAGACAAAATAGTCTACGAGGTTT**AGAGCTATGCTGTTTGAATGGTCCCAAA**ACTGAGACCA**GTCT**CGGACG**
TCCAAAGGTCTC

3. Fragment 3 (SEQ ID NO: 144), Second direct repeat: amplicon length = 452 bp
Forward primer is from 861221 to 861276 and reverse primer is from 861613 to 861594 on
20 *S. pyogenes* genomic DNA. Decamer sequence from 861246-861255 GGTCTCCATT
(SEQ ID NO: 142), which contains *BsaI* recognition sequence on the genomic DNA, was
substituted with GGTCCCAAAA (SEQ ID NO: 143) to destroy *BsaI* recognition sequence
and convert the 7th truncated direct repeat in the CRISPR array on the genome to the
canonical 2nd direct repeat sequence in this vector.

25 **GAGACCA**GTCT**CGGACGCTCCAAAGGTCTC**GTTTTAGAGCTATGCTGTTTGAATGGTCCCAAAACAACATTGCCGATGATAACTTGAG
AAAGAGGGTTAATACCAGCAGTCGGATACCTTCCTATTCTTTCTGTTAAAGCGTTTTTCATGTTATAATAGGCCAAAAGAAGAGTAGTGT
GATGGAACAAACATTTTATGATTAAGCCATATGGGGTTAAGCAAGGGGAGGTAGTTGGAGAGGTTTTACGGTGGATTGAACGCCTA
AGATTTCAGTTTAAGCGATTTCGAGCTAAGACAAGCTAGTTTCGAAATACTTGGCTAAGCACGACGAGGCCTTGGTGATAAACCTTTTGA
30 TCCTAAACTTAAAGCTTACATGACAAGTGGTCTGTTTAATTGGGATAATTCTTGGGGACTAAGGTGGT**ATCGTCCATTCCGACAGC**
ATCGCCAGTCAC

PCR conditions to generate the three fragments were:

	Fragment 1	Fragment 2	Fragment 3
5X Q5 Reaction Buffer	1 x	1 x	1 x
10 mM dNTPs	200 µM	200 µM	200 µM
10 µM Forward Primer	0.5 µM	0.5 µM	0.5 µM
10 µM Reverse Primer	0.5 µM	0.5 µM	0.5 µM
<i>S. pyogenes</i> DNA 50 - 100 ng/ul	1 ng/ul	1 ng/ul	1 ng/ul
Q5 High-Fidelity DNA Polymerase 2 U/µL (NEB)	0.04 U/µl	0.02 U/µl	0.02 U/µl
Thermocycling condition			

Initial Denaturation	98°C_60 sec	98°C_60 sec	98°C_60 sec
35 Cycles	98°C_10 sec	98°C_10 sec	98°C_10 sec
	64°C_30 sec	62°C_30 sec	62°C_30 sec
	72°C_240 sec	72°C_30 sec	72°C_30 sec
Final Extension	72°C_120 sec	72°C_120 sec	72°C_120 sec
Hold	4 °C	4 °C	4 °C

Results showing the PCR amplicons are provided in Figure 11.

Assembly of pNB100 from three PCR amplicons, tracrRNA-cas9, leader and first direct repeat, second direct repeat; plus pACYC184 digested with *EcoRV*

We employed a Gibson assembly kit from NEB (E5510) and followed the protocol provided by the manufacturer to assemble the above three PCR amplicons along with pACYC184.

The component of each fragment in the assembling reaction is shown in the following table.

0.1 pmol/μL Fragment 1	0.2 pmol
0.2 pmol/μL Fragment 2	0.2 pmol
0.2 pmol/μL Fragment 3	0.2 pmol
0.01 pmol/μL pACYC184	0.04 pmol
Fragments 1 :: Fragment 2 : Fragment 3 : vector	5 : 5 : 5 : 1
Gibson Assembly Master Mix (2X)	1x
Incubation	50 °C for 1 hr

10

2 μL of the assembly reaction was transformed to DH5α competent cells (purchased from New England Biolabs) followed by selection on chloramphenicol (35μg/mL) LB plates. The recombinants were screened by PCR using the three primer sets used for obtaining the initial three fragments. The plasmid templates giving three desired amplicons were isolated from the candidate clones and were subjected to sequence analysis.

15

The sequence of the final construct of pNB100 (SEQ ID NO: 145)

20

GAATTCGGATGAGCATTCATCAGGCGGGCAAGAATGTGAATAAAGGCCGATAAACTTGTGCTTATTTTCTTTACGG
TCTTTAAAAAGGCCGTAATATCCAGCTGAACGGTCTGGTTATAGGTACATTGAGCAACTGACTGAAATGCCTCAAAATGT

TCTTTACGATGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTCTCCATTTTAGCTTCCTTAGCTCCTGA
 AAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATCTTATTTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGAT
 CAACGTCATTTTTCGCCAAAAGTTGGCCAGGGCTTCCCGGTATCAACAGGGACACCAGGATTTATTTATCTGCGAAG
 5 TGATCTTCCGTCACAGGTATTTATTCGGCGCAAAAGTCGGTGTAGCTGCCAACTTACTGATTTAGTGATGAGT
 GTTTTGAGGTGCTCCAGTGGCTTCTGTTTCTATCAGCTGTCCCTCCTGTTTCAGCTACTGACGGGGTGGTGGCTAACGGC
 AAAAGCACCGCCGGACATCAGCGCTAGCGGAGTGATACTGGCTTACTATGTTGGCACTGATGAGGGTGTGAGTGAAGTG
 CTTTCATGTGGCAGGAGAAAAAGGCTGCACCGGTGCGTCAGCAGAATATGTGATACAGGATATATTCCGCTTCCCTCGCTC
 ACTGACTCGCTACGCTCGGTGCTTCGACTGCGGCGAGCGGAAATGGCTTACGAACGGGGCGGAGATTTCTTGGAGATGC
 10 CAGGAAGATACTTAACAGGGAAGTGAGAGGGCCGCGGCAAGCCGTTTTTCCATAGGCTCCGCCCCCTGACAAGCATCA
 CGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCTTGGCGGCTCCC
 TCGTGGCTCTCCTGTTCTTGCCTTTCCGGTTTACCGGTGTCATTCCGCTGTTATGGCCGCTTTGTCTCATTCCACGCCCT
 GACACTCAGTTCCGGGTAGGCAGTTTCGCTCCAAGCTGGACTGTATGCACGAACCCCCCTTCAGTCCGACCGCTGCGCCT
 TATCCGGTAACATCTGCTTGGAGTCCAACCCGAAAGACATGCAAAAGCACCCTGGCAGCAGCCACTGGTAATTGATTT
 15 AGAGGAGTTAGTCTGAAGTCATGCGCCGGTTAAGGCTAAACTGAAAGGACAAGTTTTGGTGACTGCGCTCCTCCAAGCC
 AGTTACCTCGGTTCAAAGAGTTGGTAGCTCAGAGAACCTTCGAAAAACCGCCCTGCAAGGCGGTTTTTTCGTTTTTCAGAG
 CAAGAGATTACGCGCAGACCAAAACGATCTCAAGAAGATCATCTTATTAATCAGATAAAATATTTCTAGATTTTCAGTGCA
 ATTTATCTCTTCAAATGTAGCACCCTGAAGTCAGCCCCATACGATATAAGTTGTAATTTCTCATGTTTGACAGCTTATCATC
 GATAAGCTTTAATGCGGTAGTTTATCACAGTTAAATTTGCTAACGCAGTCAGGCACCGTGTATGAAATCTAAATGCGCT
 CATCGTCATCTTCGGCACCCTCACCCTGGATGCTGTAGGCTAGGCTTGGTTATGCGCGTACTGCCGGCCTCTTGGCGGG
 20 ATCCAGAAGTCTTTTTCTTGCAGTGTTCCTTTCTTTTATGATAGTTTACGAAATCATCTGTGGAGCTTAGTAGGTTTA
 GCAAGATGGCAGCGCTAAATGTAGAATGATAAAAGGATTAAAGAGATTAATTTCCCTAAAAATGATAAAACAAGCGTTTT
 GAAAGCGCTTGTTTTTTGGTTTGCAGTCAGAGTAGAATAGAAGTATCAAAAAAGCACCGACTCGGTGCCACTTTTTCA
 AGTTGATAACCGACTAGCCTTATTTTAACTTGCATGCTGTTTTGAAATGGTTTCAACACAGATTATTTTATACTTTTATA
 25 ACAAATTAATCAAGGAGAAATTTCAAAGAAATTTATCAGCCATAAAACAATCTTAATACTATAGAATGATAACAAATAAA
 CTACTTTTTAAAGAATTTTGTGTTATAATCTATTTATTTAAGTATTTGGGTAATATTTTTTGAAGAGATATTTTGAAA
 AAGAAAAATTAAGCATATTTAACTAATTTTCGAGGTCATTTAACTATTTATGAAATCATCAAACTCATTATGGATTTA
 ATTTAAACTTTTTATTTTAGGAGGCAAAAATGATATAAGAAATACTCAATAGGCTTAGATATCGGCACAAATAGCGTCCGA
 TGGGCGGTGATCAGCTGATGAATATAAGGTTCCGTCATAAAAGTTCAAGGTTCTGGGAAATACAGACCGCCACAGTATCAA
 30 AAAAAATCTTATAGGGGCTCTTTTATTTGACAGTGGAGAGACAGCGGAGCGACTCGTCTCAAAACGGACAGCTGATAGAA
 GCTATACAGCTCGGAGAAATGATTTTCTTATCTACAGGAGATTTTTCAAATGAGATGGCGAAAGTAGATGATAGTTTC
 TTTTCATCGACTTGAAGAGTCTTTTTTGGTGGAGAAAGACAAGAGCATGAACGTCATCTATTTTGGAAATATAGTAGA
 TGAAGTTGCTTATCATGAGAAATATCCAACATCTATCATCTGCGAAAAAATTTGGTAGATTCTACTGATAAAGCGGATT
 TGGCTTAATCTATTTTGGCCTTAGCGCATATGATTAAAGTTTCGTGGTCATTTTTGATTGAGGGAGATTTAAATCCTGAT
 35 AATAGTGATGTGGACAACTATTTATCCAGTTGGTACAACTACAATCAATTATTTGAAGAAAACCCATTAACGCAAG
 TAGGTAGATGCTAAAGCGATTCTTTCTGACAGTATGATTAATCAAGACGATTAGAAAAATCTCATTGCTCAGCTCCCCG
 GTGAGAAGAAAAATGGCTATTTGGGAATCTCATTGCTTTGTCATTGGGTTTGACCCCTAATTTTAAATCAAATTTTGAT
 TTGGCAGAAGATGCTAAATTACAGCTTTCAAAGATACTTACGATGATGATTAGATAATTTATTTGGCGCAAAATGGAGA
 TCAATATGCTGATTTGTTTTTGGCAGCTAAGAATTTATCAGATGCTATTTTACTTTTTCAGATATCCTAAGAGTAAATACG
 40 AAATAACTAAGGCTCCCTATCAGCTTCAATGATTAAACGCTACGATGAACATCATCAAGACTTGACTCTTTTAAAGCT
 TTAGTTTCGACAACAACCTCCAGAAAAGTATAAAGAAATCTTTTTTGATCAATCAAAAAACGGATATGCGAGTTATATTGA
 TGGGGGAGCTAGCCAAGAAGAAATTTTATAAATTTATCAAAACCAATTTTAGAAAAATGGATGGTACTGAGGAATTTTGG
 TGAAACTAAATCGTGAAGATTTGCTGCGCAAGCAACGAGCTTTGACACCGGCTCTATTTCCCATCAAATTCAGCTGGGT
 GAGCTGCATGCTATTTTGAAGAAGACAAGAGACTTTTATCCATTTTTTAAAGACAATCGTGAGAAGATTGAAAAATCTT
 45 GACTTTTTCGAATTCCTTATTATGTTGGTCCATTGGCGCGTGGCAATAGTCGTTTTGCATGGATGACTCGGAAGTCTGAAG
 AAACAATTACCCCATGGAATTTTGAAGAAGTTGTCGATAAAGGTGCTTCAGCTCAATCATTTATTTGAACGCATGACAAAC
 TTTGATAAAAAATCTTCCAAATGAAAAAGTACTACCAAAACATAGTTTGTCTTATGAGTATTTTACGGTTTATAACGAAT
 GACAAAGGTCAAATATGTTACTGAAGGAATGCGAAAACAGCATTTCTTTTCAGGTGAACAGAAGAAAGCCATTGTTGATT
 TACTCTTCAAAACAAATCGAAAAGTAACCGTTAAGCAATTAAGAAGATTAATTTCAAAAAATAGAATGTTTTGATAGT
 GTTGAAATTTTCCAGGAGTTGAAGATAGATTTAATGCTTCATTAGGTACCTACCATGATTTGCTAAAAATTTAAGATAA
 50 AGATTTTTTGGATAATTAAGAAAATGAAGATATCTTAGAGGATATTGTTTTAACATTGACCTTATTTGAAGATAGGGAGA
 TGATTGAGGAAAGACTTAAACATATGCTCACCTCTTTGATGATAAGGTGATGAAACAGCTTAAACGTCGCGCTTATACT
 GGTGGGGACGTTTGTCTCGAAAATGATTAAATGGTATTAGGATAAGCAATCTGGCAAAACAAATTTAGATTTTTTGAA
 ATCAGATGGTTTTGCCAATCGCAATTTTATGCAGCTGATCCATGATGATAGTTTGACATTTAAAGAAGACATTCAAAAAG
 CACAAGTGTCTGGACAAGGCGATAGTTTACATGAACATATTGCAAAATTTAGCTGGTAGCCCTGCTATTAAGAAAGGTAT
 55 TTACAGACTGTAAAAGTTGTTGATGAATTTGGTCAAAGTAATGGGGCGGCATAAGCCAGAAAAATATCGTTATTGAAATGGC
 ACGTGAATAATCAGACAACTCAAAGGGCCAGAAAAATTCGCGAGAGCGTATGAAACGAATCGAAGAAGGTATCAAAGAAT
 TAGGAAGTCAGATTCTTAAGAGCATCCTGTTGAAAATCAATCAATTTGCAAAATGAAAAGCTCTATCTCTATTATCTCCAA
 AATGGAAGAGACATGTATGTGGACCAAGAATTAGATATTAATCGTTTTAAGTGATTATGATGTCGATCACATTGTTCCACA
 AAGTTTCTTAAAGACGATTCAATAGACAATAAGGTCTTAACCGGTTCTGATAAAAAATCGTGGTAAATCGGATAACGTTT
 60 CAAGTGAAGAAGTAGTCAAAGAGATGAAAACTATTGGAGACAACCTTCTAAACGCCAAGTTAATCACTCAACGTAAGTTT
 GATAATTTAACGAAAGCTGAACGTGGAGGTTTGGTGAACCTTGATAAAGCTGGTTTTATCAAACGCCAATTGGTTGAAAC
 TCGCCAAATCACTAAGCATGTGGCACAAATTTTGGATAGTCGCATGAATACTAAATACGATGAAATGATAAATCTATTC
 GAGAGGTTAAAGTGATTACCTTAAATCTAAATTAGTTTCTGACTTCCGAAAAGATTCCAATCTATAAAGTACGTGAG

ATTAACAATTACCATCATGCCCATGATGCGTATCTAAATGCCGTCGTTGGAACGCTTTGATTAAAGAAATATCCAAAAC
 TGAATCGGAGTTTGTCTATGGTGATTATAAGTTTATGATGTTTCGTAATAATGATTGCTAAGTCTGAGCAAGAAATAGGCA
 AAGCAACCGCAAAATATTTCTTTTACTCTAATATCATGAACCTCTTCAAACAGAAATTACACTTGCAAATGGAGAGATT
 CGCAAACGCCCTCTAATCGAACTAATGGGGAACCTGGAGAAATTGCTGGGATAAAGGGCGAGATTTTGCACAGTGGC
 5 CAAAGTATTGTCATGCCCCCAAGTCAATATTTGTCAGAGAAACAGAAAGTACAGACAGGCGGATTTCTCCAGGGAGTCAATTT
 TACCAAAAAGAAATTCGGACAAAGCTTATTGCTCGTAAABAGACTGGGATCCAAAABAAATATGGTGGTTTGTAGTCCCA
 ACGGTAGCTTATTTCAGTCCCTAGTGGTTGCTAAGGTGGAAAAAGGGAATCGAAGAGTTAAATCCGTTAAAGAGTTACT
 AGGGATCACAAATTATGAAAGAGTTCCTTTGAAAAAATCCGATTGACTTTTGAAGCTAAAGGATATAAGGAAGTTA
 10 AAAAAAGCTTATCATTAACCTACCTAATATAGTCTTTTGGAGTTAGAAAACGGTCTGTAACGGATGCTGGCTAGTGGC
 GGAGAATTACAAAAGGAAATGAGCTGGCTCTGCCAAGCAAAATATGTGAATTTTATATTAGCTAGTCATTATGAAAA
 GTTGAAGGGTACTCCAGAAGATAACGAACAAAACAATTGTTTGGGAGCAGCATTAAGCAATTATTAGATGAGATTATTG
 AGCAAAATCAGTGAATTTTCTAAGCGTGTATTCTTAGCAGATGCCAATTAGATAAAGTTCCTAGTGCATATAACAAACAT
 AGAGACAAACCAATACGTGAACAAGCAGAAAATATTATTCAATTATTACGTTGACGAATCTTGGAGCTCCCGCTGCTTT
 15 TAAATATTTTGATACAACAATTGATCGTAAACGATATACGCTACAAAAGAAGTTTATAGATGCCACTCTTATCCATCAAT
 CCATCACTGGTCTTTATGAAACACGCATTGATTTGAGTCAGCTAGGAGGTGACTGATGGCCACGTGAACATATGATTTT
 CCGCAGTATATTTTAGATGAAGATTATTTCTTAATAACTAAAAATATGGTATAATACTCTTAAATAATCGAGTAATACAG
 GGGCTTTTCAAGACTGAAGTCTAGCTGAGACAAATAGTGCGATTACGAAATTTTATAGACAAAATAGTCTACGAGGTTT
 TAGAGCTATGCTATTTTGAATGGTCCCAAACTGAGACCAGTCTCGGACGTCCAAAGGTCTCGTTTATAGCTATGCTGT
 20 TTTGAATGGTCCCAAAACACATTGCCGATGATAACTTGAGAAAGAGGGTTAATACCAGCAGTCGGATACCTTCCATATTC
 TTTCTGTAAAGCGTTTTCATGTTAATAGGCAAAAGAAAGTAGTGTGATGGAACATACATTTTATGATTAAAGCCA
 TATGGGGTTAAGCAAGGGGAGGTAGTTGGAGAGGTTTACGGTGGATTGAACGCCTAAGATTTACGTTTAAAGCATTGCA
 GCTAAGACAAGCTAGTTGCAAACTACTGGCTAAGCAGCAGCAGGCGCTTGGTGATAAACCTTTTATCCTAACTTAAAGC
 TTACATGACAAGTGGTCTGTTTAAATGGGATAATTCTTGGGACTAAGGTGGTATCGTCCATTCCGACAGCATCGCCA
 25 GTCACTATGGCGTGCTGCTAGCGCTATATGCGTTGATGCAATTTCTATGCGCACCCGTTCTCGGAGCACTGTCCGACCGC
 TTTGGCGCGCGCCAGTCTGCTCGCTTCGCTACTTGGAGCCACTATCGACTACGCGATCATGGCGACCACACCCGTCCT
 GTGGATCCTCTACGCCGGACGCATCGTGGCGGCATCACCAGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACA
 TCACCGATGGGGAAGATCGGGCTCGGCCACTTCGAGCGCTCATGAGCGCTTGTTCGGCGTGGGTATGGTGCAGGCCCGCTG
 GCGGGGGAGTGTGGGCGCCATCTCCTTGCAATGCACCATTCCTTGCGCGCGGGTGTCAACGGCCTCAACCTACTACT
 GGGCTGCTTCTAATGCAGGAGTCGCATAAGGGAGAGCGTGGACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCT
 30 TCCGGTGGGCGGGGCGATGACTATCGTCGCCGCACTTATGACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCG
 GCAGCGCTCTGGGTCAATTTTCGGCGAGGACCGCTTTCGCTGGAGCGCGACGATGATCGGCTGTGCTTGGCGTATTCCG
 AATCTTGACACGCCCTCGCTCAAGCCTTCGTCAGTGGTCCCGCCACCAACGTTTCGGCGGAGAAGCAGGCCATTATCGCCG
 GCATGGCGGGCGACGCGCTGGGCTACGCTTGTCTGGCGTTTCGCGACGCGAGGCTGGATGGCCTTCCCCATTATGATTCTT
 CTCGCTTCCGGCGGCATCGGGATGCCGCGTTCGAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCT
 35 TCAAGGATCGCTCGCGCTCTTACAGCCTAAGCTTCGATCTGAGACCGCTGATCGTCACGGCGATTATGCGCCCTGAGCG
 CGAGCACATGGAACGGGTTGGCATGGATTGTAGGCGCGCCCTATACCTTGTCTGCCTCCCCGCGTTGCGTTCGCGGTGCA
 TGGAGCGGGCCACCTCGACCTGAATGGAAGCGCGGCACCTCGCTAACGGATTACCACTCCAAGAATTGGAGCCAAT
 CAATCTTTCGGGAGAATGTGTAATGCGCAACCAACCTTGGCAGAATATCCATCGGCTCCGCCATCTCCAGCAGCCG
 CACGCGGCGCATCTCGGGCAGCGTTGGGTCTGGCCACGGGTGCGCATGATCGTCTCTGCTTGGAGACCCGGCTAG
 40 GCTGGCGGGGTTGCCTTACTGGTTAGCAGAATGAATCACCGATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAAACG
 TCTGCGACCTGAGCAACAACATGAATGGTCTTCGGTTTCGGTTCGTAAGTCTGGAACCGGGAAGTCCCTTACGTC
 CTGCTGAAGTTGCCCGCAACAGAGAGTGGAAACCAACCGGTGATACCACGATACATGACTGAGAGTCAACGCCATGAGCG
 GCCTCATTTCTTATTCTGAGTTACAACAGTCCGACCCGCTGTCCGGTAGCTCCTTCCGGTGGGCGCGGGCATGACTATC
 45 GTCGCCGCACTTATGACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCCCAACAGTCCCCCGGCCAC
 GGGGCTGCCACCATAACCCAGCGCAACAAAGCGCCCTGCACCATTATGTTCCGGATCTGCATCGCAGGATGCTGCTGGC
 TACCCTGTGGAACACCTACATCTGTATTAACGAAGCGCTAACCGTTTTTATCAGGCTCTGGGAGGCAGAATAAATGATCA
 TATCGTCAATTAATACCTCCACGGGGAGAGCCTGAGCAAACTGGCCTCAGGCATTTGAGAAGCACACGGTCACACTGCTT
 CCGGTAGTCAATAAACCGGTAAACAGCAATAGACATAAGCGGCTATTTAACGACCTGCCCTGAACGACGACCGGGT
 50 GAATTTGCTTTTGAATTTCTGCCATTATCCGCTTATTATCACTTATTCAGGCGTAGCACAGGCGTTTAAAGGCACCA
 TAACTGCCTTAAAAAATTACGCCCCCGCTGCCACTCATCGCAGTACTGTTGTAATTCATTAAGCATTCTGCCGACATG
 GAAGCCATCACAGACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTGCGCTTGGGTATAAATTTGCCCA
 TGGTGAAAAACGGGGCGAAGAAGTTGTCCATATTGGCCACGTTTAAATCAAACTGGTGAAACTCACCCAGGGATTGGCT
 GAGACGAAAAACATATTCTCAATAAACCTTTAGGGAATAGGCCAGGTTTTTACCCTAACACGCCACATCTTTCGAATA
 55 TATGTGTAGAACTGCCGAAATCGTCTGTTTACTTCCAGAGCGATGAAAACGTTTCAGTTTGGCTCATGGAACCGG
 TGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCCTTTTCATTGCCATACG

The total number of nucleotides is 9578 bp. The backbone vector pACYC184 sequence is italicised, sequence positions are numbered from G of *EcoRI* site (GAATTC) underlined.

tracrRNA is located at nucleotide No. from 1889 to 1974 indicated bold letters, Cas9

60 initiation and termination codons are indicated by bold three letters, starting at

nucleotide No. 2270 and ending at 6376 followed by leader sequence 6462-6556 indicated by italicised bold letters, first and second direct repeat sequences are underlined, between which spacer cloning region (30 mer) is located. This spacer cloning region contains two inverted *Bsa*I sites indicated by bold italicised letters 5'-GAGACC-3' and 5'-GGTCTC-3' for
 5 creating 5' four bases protruding spacer cloning sites 5'-GTTT-3' and 5'-TTTT-3', respectively and one unique *Aat*II (5'-GACGTC-3') site also indicated by bold italicised to reduce self-ligation in the event of incomplete *Bsa*I digestion. Note the transition and transversion base changes G6573A, A6779T that were detected by Sanger sequencing and are shown in bold letters, respectively. However, these point mutations do not affect
 10 the CRISPR-Cas9 activity, which will be shown in the later section. The two unique sites, *Sa*I (GTCGAC) and *Xba*I (TCTAGA) highlighted in bold italicised letters are utilised to isolate the CRISPR/Cas9 construct for cloning into M13mp18.

A plasmid map of pNB100 is shown in Figure 12.

15 The desired spacer sequence can be cloned in the clockwise direction between *Bsa*I sites. This vector contains the p15A origin at 1393-848 and *cat* (chloramphenicol resistant) gene at 219-9137. Cutting positions of each restriction enzyme, indicated in the parentheses, refer to the position of the 5' cutting sites on the top strand within the recognition sequence.

20

Construction of pNB102

pNB100 was digested with *Bsa*I and *Aat*II followed by purification using Agencourt ampure beads. The spacer sequence CR30 was employed from the discussion in B. Selection of
 25 spacer sequence from the target sequence in the Materials and Methods section above. The CR30 sequence is as follows :

5' -AAACACTTTAAAAGTGCTCATCATg (SEQ ID NO: 117)
 tgaaattttcaccgagtagtacAAAA-5' (SEQ ID NO: 118)

30

This double-stranded DNA cassette is generated by denaturation at 95 C for 1 min and re-annealing at -1 degree every min to 30 C in the 1 x T4 ligase buffer (50 mM Tris-HCl(pH 7.5 at 25 C), 10 mM MgCl₂, 1 mM ATP 10 mM DTT) plus 50 mM NaCl following the kinase reaction to add a phosphate moiety in the 5' terminus of each oligo. The annealed cassette
 35 contains 5' protruding four base compatible bases on both ends for the sites created on pNB102 by *Bsa*I digestion. This CR30 cassette was ligated to pNB100 by T4 DNA ligase and transformed to DH5α competent cells (purchased from New England Biolabs). The

transformants were selected on chloramphenicol LB plate and were screened by PCR with the bottom sequence of the CR30 cassette as a reverse primer and a forward primer CF1: 5'-acgttgacgaatcttgagc, which anneals at 6209-6228 region on the recombinant plasmid to generate 409 bp PCR amplicon. PCR positive clones were sequenced to confirm the

- 5 CR30 spacer sequence and this recombinant clone is designated as pNB102 and used for *in vitro beta lactamase*-gene disruption experiments. CR30 spacer anneals to the sense strand of *beta lactamase*-gene and cleaves the phosphodiester bonds between 188th and 189th nucleotide on the sense and antisense strand.

10

Construction of M13mp18::NB102

pNB102 was digested with unique restriction sites *SalI* and *XbaI* to generate two fragments 6044 bp and 3524 bp. The fragment length was calculated from the 5' end of the restricted sites in the top strand within the restriction recognition sites. The 6044 bp fragments

- 15 containing CRISPR locus with CR30 spacer sequence in the CRISPR array was separated from the 3524 bp fragment and purified on the preparative 1 % agarose gel. M13 mp18 was digested with *SalI-XbaI*, followed by Agencourt ampure purification to remove the six bases *SalI-XbaI* fragment from the reaction. These two purified fragments were combined and ligated by T4 DNA ligase and transformed to DH5 α F'lq competent cells (purchased
- 20 from New England Biolabs). Transformed cells were plated along with freshly grown DH5 α F'lq cells as a phage indicator and IPTG/X-gal solution as a blue-white colour indicator with molten top agar to LB plate. White plaques collected from the lawn were screened by PCR for the presence of the CR30 spacer sequence. The correct phage constructs obtained were purified by two times single plaque isolation. The entire sequence
- 25 length of the final construct is 13288 bp. This spacer CR30 positive recombinant is designated as M13mp18::NB102 and was used for the *bla*-gene inactivation experiments mediated by M13 phage delivery.

30 Delivery of CRISPR-Cas9 constructs to bacteria

Having constructed the CRISPR-Cas9 plasmid pNB100 and the derivative plasmid pNB102 carrying a spacer insertion targeted against the beta-lactamase (*bla*) genes encoded by the bacterial transposable elements Tn1 and Tn3, we then sought to demonstrate, using three delivery methods, (i) plasmid conjugation, (ii) plasmid DNA transformation, (iii)

- 35 bacteriophage infection, that bacterial cells carrying copies of the CRISPR-Cas9 construct with *bla*-spacer insertion would be unable to grow in the presence of the beta-lactam antibiotic ampicillin.

Constructs that are able to inactivate target genes, including antibiotic resistant genes, via the CRISPR-Cas system, and which are an aspect of the present invention are also referred to herein as “Nemesis symbiotics”.

5

“Nemesis symbiotic activity” (NSA) assay by plasmid conjugation: a prophylaxis experiment

We demonstrate here that Nemesis symbiotics can prevent the spread of antibiotic resistance by inhibiting conjugal transfer of conjugative plasmids carrying antibiotic
 10 resistance genes from a donor cell to a recipient cell carrying the Nemesis symbiotics. To do this we mated a recipient cell carrying the Nemesis symbiotics with a donor cell carrying a conjugative plasmid, plus a multicopy mobilisable plasmid carrying the *bla* gene encoding ampicillin resistance. In a successful mating, the conjugative plasmid will transfer itself plus the mobilisable plasmid carrying ampicillin resistance to the recipient. Exconjugants may be
 15 selected for resistance to both chloramphenicol present on a non-mobilisable plasmid in the recipient and ampicillin received from the donor. Successful Nemesis symbiotic activity will reduce the efficiency of transfer of ampicillin resistance.

The recipient cell DH5 α (F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG
 20 Φ 80dlacZ Δ M15 Δ (lacZYA-argF)U169, hsdR17(rK- mK+), λ -) was purchased from New England Biolabs and transformed with the plasmids pNB100 or pNB102 or pACYC184, where plasmids encode chloramphenicol resistance and both pNB100 and pNB102 carry CRISPR-Cas9 but only pNB102 carries the spacer sequence targeted against the beta-lactamase gene. The plasmid pACYC184 is the non-mobilisable parent plasmid used for
 25 the construction of pNB100 and pNB102 as described above.

The donor strain JA200 (F+ thr-1, leu-6, DE(trpE)5, recA, lacY, thi, gal, xyl, ara, mtl) also carrying plasmid pNT3 is described by Saka et al. DNA Research 12, 63–68 (2005). The plasmid pNT3 is a mobilisable plasmid carrying the *bla* gene of Tn1.

A single colony of the donor JA200 pNT3 was picked from a Luria broth (LB) plate
 30 containing 100 μ g/mL Ampicillin and grown shaking at 37°C overnight in 1mL LB medium with 100 μ g/mL Ampicillin. A single colony each of the recipients, DH5 α pNB100 and DH5 α pNB102 was picked from a LB plate containing 35 μ g/mL Chloramphenicol and grown shaking at 37°C overnight in 1mL LB with 35 μ g/mL Chloramphenicol. To wash cells to remove antibiotics, 50 μ L of cells were added to 1mL LB in Eppendorf tubes and

centrifuged 60 sec at 12500 rpm. Cells were resuspended in 50µL LB. To set up the mating JA200 pNT3 was spotted onto an LB plate, then 2µL of each DH5α carrying pNB100 and pNB102 were added to this spot. Separate 2µL spottings of donor and recipients were also performed (i.e. not mated). Plates were incubated at 37 °C for 4 hours. Cells were removed
 5 resuspended in LB and 100µL plated on LB plates containing both 100 µg/mL Ampicillin and 35 µg/mL Chloramphenicol (LB ApCm). 100µL of 10,000 fold (10^{-4}) dilutions were also plated on LB plates and incubated at 37 °C overnight. The resultant colonies were counted as shown in the Table below.

Cells	LB ApCm plates	LB plates 10^{-4} dilution	Nemesis symbiotic activity
JA200 pNT3 x DH5α pNB100	Confluent	Approx. 500	Negative
JA200 pNT3 x DH5α pNB102	37	Approx. 500	Positive
JA200 pNT3 x DH5α pACYC184	Confluent	Approx. 500	Negative
JA200 pNT3	0	Not done	Not applicable
DH5α pNB100	0	Not done	Not applicable
DH5α pNB102	0	Not done	Not applicable
DH5α pACYC184	0	Not done	Not applicable

10

Photographs in Figure 13 show platings of the matings between: (A) JA200 pNT3 x DH5α pNB100 (as expected lacking Nemesis symbiotic activity); and (B) JA200 pNT3 x DH5α pNB102 (showing Nemesis symbiotic activity).

- 15 The 10^{-4} dilution plated on LB plates, gave approximately 500 colonies a count of 5×10^7 cells per mL in the mated cell suspension and for the JA200 pNT3 x DH5α pNB102 only 3.7×10^2 cells/mL were able to grown on the LB Ap100Cm35 plates. Thus assuming half the cells are recipients/ex-conjugants then: 3.7×10^2 cells /mL divided by 2.5×10^7 gives a mating efficiency for the recipient carrying pNB102 of $1.2 \times 5 \times 10^{-5}$

The experiment demonstrated a significant reduction in CmRApR exconjugants in matings with DH5 α carrying pNB102 versus pNB100 x JA200 pNT3.

- 5 In order to measure the relative mating efficiencies more accurately, after a liquid mating cells were plated on LB plates to titre all cells, LB Ap100 plates to titre donors plus exconjugants, LB Cm plates to titre recipients plus exconjugants and LB Ap100Cm35 to titre exconjugants only.
- 10 For the liquid mating overnight cultures of 10 μ L of JA200 pNT3 were mixed with 10 μ L of recipients DH5 α pNB100 or DH5 α pNB102 200 μ L of LB added and tubes incubated overnight at 37°C. Mating mixtures were diluted 10⁻¹, 10⁻³, 10⁻⁵ in LB and 50 μ L of dilutions plated on LB, LB Ap100Cm35, LB Ap100 and LB Cm35 plates and plates incubated overnight at 37°C. The table below summarises the cell titres obtained.

15

Mated with JA200 pNT3	LB All cells	LB Ap Cm Exconjugants	LB Cm recipients and exconjugants	LB Ap donors and exconjugants	mating effc/donor	mating effc/recipient
pNB100	4.14 x 10 ⁸	2.80 x 10 ⁷	1.40 x 10 ⁸	2.64 x 10 ⁸	1.06 x 10 ⁻¹	2.00 x 10 ⁻¹
pNB102	5.16 x 10 ⁸	7.20 x 10 ³	1.78 x 10 ⁸	3.82 x 10 ⁸	1.88 x 10 ⁻⁵	4.04 x 10 ⁻⁵

- The number of cells on LB Cm plus LB Ap plates should equal the number of cells on LB plates. For pNB100 1.40 x 10⁸ (Cm plates) plus 2.64 x 10⁸ (Ap plates) = 4.04 x 10⁸ which agrees very well with 4.14 x 10⁸ on LB plates. For pNB102 1.78 x 10⁸ (Cm plates) plus 3.82 x 10⁸ (Ap plates) = 5.6 x 10⁸ which agrees very well with 5.16 x 10⁸ on LB plates.
- 20

- In conclusion, the data show that after overnight mating in liquid culture, there is a 5,000 fold reduction in mating efficiency per recipient comparing pNB102 with the spacer to pNB100 lacking the spacer.
- 25

NSA Assay by plasmid transformation

In this experiment, we demonstrate that introduction of Nemesis symbiotics to recipient cells by DNA transformation inactivates antibiotic resistance in the transformants.

- 5 In order to obtain a tester strain, DH5 α competent cells purchased from New England Biolabs were transformed with pBR322 (carrying the *bla* gene derived from Tn3) and selected on LB Ap100 plates. Competent cells of the derived strain DH5 α pBR322 were then prepared using the CaCl₂ protocol 25 (1.116) as described by Sambrook and Russell in Molecular Cloning: A Laboratory Manual (3rd Edition, 2001) and subsequently
- 10 transformed with plasmids pNB100, pNB102 and pACYC184 with selection for CmR. Transformant colonies were then picked onto LB Cm35 and LB Ap100 plates. Primary transformants were replica toothpicked onto both LB Cm35 and LB Ap100 plates and incubated overnight at 37 °C.
- 15 The results, depicted in Figure 14, show that all colonies toothpicked from DH5 α pBR322 transformed by pNB100 (lacking the *bla* gene target spacer sequence) remain resistant to ampicillin. In contrast all colonies toothpicked from DH5 α pBR322 transformed by pNB102 have lost ampicillin resistance, so demonstrating Nemesis symbiotic activity.
- 20 The experiments above do not give a value for the fraction of primary transformants where NSA has inactivated the *bla* gene. To address this, single colonies from the primary transformants were picked into 1mL LB and diluted 10⁻³ in LB. Then 100 μ L plated onto plates as follows, and results scored. The results showed that following transformation of DH5 α pBR322 with pNB102 fewer than 10⁻⁶ cells retain ApR. Nemesis symbiotic activity
- 25 is very efficient.

NSA Assay by bacteriophage M13 infection

In this experiment, we demonstrate that introduction of Nemesis symbiotics to recipient cells by bacteriophage infection inactivate antibiotic resistance in the transformants. We

- 30 chose the male-specific filamentous phage M13 as the delivery agent for the Nemesis symbiotic construct. An M13 derivative M13mp18::NB102 carrying and the Cas9 CRISPR plus *bla* gene target spacer region of pNB102 was used to deliver the Nemesis symbiotic by infection of an F⁺ strain, JA200, carrying ampicillin resistance on the plasmid pNT3. 0.2mL of a fresh culture of this strain was added to 3 mL of LB top agar (Luria broth with
- 35 0.7% agar) and poured onto an LB plate. Then 2 μ L of phage stocks of M13mp18::NB102 (10⁸ pfu/mL) and as a control M13mp18 were spotted onto the lawn and the plate was

incubated 8 hours at 37°C. Plaques were picked into 1.5 mL LB and grown shaking o/n at 37°C. A control strain DH5α lacking ampicillin resistance was also cultured overnight from a single colony picked into 1.5 mL LB.

5 Nitrocefin assay for beta lactamase activity was performed:

1mL of the culture of cells was centrifuged for 60 sec at 12,500 rpm in microfuge then 2μL of stock nitrocefin (10mg/mL in DMSO) was added to 1mL of cell supernatant and absorbance of the degradation product of nitrocefin was measured at 482 nm in a spectrophotometer several time points after addition of nitrocefin. The Table below

10 summarises the results.

Strain	30 sec	60 sec	2 min	5 min
JA200 pNT3 infected by M13mp8	0.5	0.64	0.73	0.79
JA200 pNT3 infected by M13mp8::NB102	0.08	0.09	0.11	0.15
DH5α	0.09	0.07	0.06	0.05

The experiments reported above provide the proof-of-concept that, in the model organism, *Escherichia coli*, DNA constructs carrying the Cas9 CRISPR region plus a spacer region with sequences directed against a target region of the beta-lactamase gene can inactivate

15 ampicillin resistance when delivered by naked DNA transformation and bacteriophage infection as well as prevent transfer of ampicillin resistance by plasmid conjugation. It is apparent that Nemesis symbiotics of the invention can be applied to pathogenic bacteria and for other antibiotic resistance genes.

20

Example 3

The aim of Example 3 is to extend the proof-of-concept for resurrection of antibiotic efficacy by introduction of a CRISPR/Cas9 construct in a pathogenic bacterial strain of *Klebsiella pneumoniae*. Resistance to a newer class of beta-lactam antibiotics, the Carbapenems has

25 emerged in Enterobacteriaceae by the acquisition of new *bla* genes that encode beta-lactamases able to degrade even the Carbapenems. *Klebsiella pneumoniae* strains with resistance to Carbapenems, KPC are important causes of morbidity and mortality among hospital-acquired and long-term care—associated infections. Noteworthy is the very high mortality (around 30–70%) among patients with bacteraemia or pulmonary infections.

Example 3 shows that a *Klebsiella pneumoniae* carrying a beta-lactamase (*bla*) gene conferring resistance to the beta-lactam antibiotic, ampicillin become sensitive to ampicillin following introduction of a modified CRISPR/Cas9 construct targeted against the *bla* gene.

- 5 The same CRISPR/Cas9/anti-*bla* construct used in Example 1 or Example 2 is used and is delivered to *Klebsiella pneumoniae* by conjugation with a donor strain carrying a conjugative plasmid with CRISPR/Cas9/anti-*bla*. Ex-conjugant recipient *Klebsiella pneumoniae* carrying the plasmid with CRISPR/Cas9/anti-*bla* construct is selected for on appropriate media with counter-selection against the donor cell and *Klebsiella pneumoniae* cells that failed to
10 receive the plasmid.

As for Example 1 and Example 2, efficacy of the CRISPR/Cas9/anti-*bla* construct is evaluated by comparison to a negative control of a *Klebsiella pneumoniae* ex-conjugant strain that received a plasmid with CRISPR/Cas9 (*i.e.* lacking the anti-*bla* region). As in

- 15 Example 1 and Example 2, the beta-lactamase activity can be detected by nitrocefin to count white versus red colonies. Also as in Example 1 and Example 2, alternatively beta lactamase activity is seen directly challenging the bacteria on the LB plate with/without ampicillin and measuring the fraction of the ampicillin sensitive colonies versus ampicillin resistant ones.
- 20 The identical experiment to that described above is also evaluated using instead a CRISPR/Cas9/anti-*blaCb*, where the CRISPR/Cas9/anti-*blaCb* construct carries a region, anti-*blaCb*, targeted against a *bla* gene encoding a beta-lactamase able to break down carbapenems and present in that particular *Klebsiella pneumoniae* strain.

25 **Example 4**

Example 4 provides delivery routes for the therapeutic constructs. These routes all apply to veterinary as well as human applications to be delivered orally, topically, probiotically and for use in surgical irrigation fluids and wound dressings.

- 30 Orally: Phage containing assassin construct as the active ingredient may be administered orally as a stabilised therapeutic preparation: either - administered before antibiotic therapy or administered in the form of an adjuvant complexed to an antibiotic. Conjugative plasmids containing assassin construct as the active ingredient may be administered as a culture of commensal bacteria carrying these plasmids in order to transmit these plasmids to gut flora
35 thereby generating prophylactic protection against future infection with antibiotic resistant bacterial pathogens.

- Topically: Phage containing assassin construct as the active ingredient may be administered topically as a stabilised medication (ointment, spray, powder etc): either - administered before antibiotic topical medication; or administered in the form of a complex with an
- 5 antibiotic medication. Topical application of conjugative plasmids containing assassin construct as the active ingredient may be via a stabilised culture of commensal bacteria carrying these plasmids in order to transmit the plasmids to gut flora thereby generating prophylactic protection against future infection with antibiotic resistant bacterial pathogens.
- 10 Probiotically: Phage containing assassin construct as the active ingredient may be administered probiotically as a stabilised culture of commensal bacteria (e.g. *Lactobacillus* spp) carrying these plasmids in order to transmit the plasmids to gut flora thereby generating prophylactic protection against future infection with antibiotic resistant bacterial pathogens. One example of this may be the probiotic, prophylactic administration of such a preparation
- 15 to patients in settings with a high risk of infection with antibiotic resistant bacterial pathogens such as hospitals, care homes, schools, transplantation centres etc. Another example may be the administration of such a preparation to livestock via animal feeds, thereby limiting the rise and horizontal transmission of antibiotic resistant bacteria. The use of the present invention in livestock thus represents one means for (indirect) prophylactic treatment of
- 20 antibiotic resistant bacteria in humans.

- Surgical irrigation fluids: Phage containing assassin construct as the active ingredient may be added to surgical irrigation fluids and also sprays for disinfecting fomites. Stabilised commensal bacterial cultures containing conjugative plasmids incorporating assassin
- 25 constructs may be added as coatings to surgical wipes and to wound dressings.

Example 5

- In this example, a construct is designed to include multiple RNA guide molecules where
- 30 each RNA guide molecule is transcribed by its own promoter.

- Figure 16 exemplifies a set of spacer sequences that we have identified encoding 20 guide RNA molecules targeted against 117 different *bla* genes identified in the NCBI ARDB database for *Klebsiella pneumoniae*. The beta lactamase gene type, spacer sequence and
- 35 antibiotic resistance profile in *Klebsiella pneumoniae* obtained from NCBI ARDB database are shown.

Beta lactamase gene sequences were collected from the ARDB database with the keyword *Klebsiella pneumoniae*. Redundant sequences were removed and unique sequences used for multiple sequence alignment using web program Clustal Omega. One canonical sequence was chosen from each cluster and the 20 nt spacer sequences predicted by the web program Jack Lin's CRISPR/Cas9 gRNA finder collected. The spacer sequence was chosen to maximise the ratio of the proto-spacer sequence found in the sequences belonging to the same branch. Each of the example spacer sequences shown in the 4th column has the capability to disrupt the genes in the third column.

- 10 Beta lactamase genes used in this analysis are: SHV-a = 1, 2, 2a, 5, 5a, 11, 12, 14, 26, 27, 28, 31, 33, 38, 43, 44, 48, 55, 56, 60, 61, 62, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 85, 89, 92, 98, 99, 101, 103, 106, 107, 108, 109, CTXM-b = 1, 3, 10, 12, 15, 22, 32, 54, 60, 62, 68, CTXM-c = 13, 14, 16, 18, 19, 24, 26, CTXM-d = 2, 35, 59, CTXM-e = 26, 63, TEM-f = 1, 1b, 3, ESBL, 139, KPC-g = 1, 2, 3, 4, OKP-h = A11, A12, A16, A17, B13, B-SB613, 6, LEN-
15 i = 2, 17, 18, 19, 20, 21, 22, 24, GES-j = 1, 3, 4, 5, VIM-a = 1, 2, 4, 12, 19, IMP-b = 4, 8, CMY-a = 2, 4, 25, 31, 36, LAT-b = 1, 2, CMY-c = 1, 8b, 10, 19, FOX-d = 1, 5, 7, OXA-a = 1, 30, 47, OXA-2, OXA-9, OXA-b = 10, 17.

Beta lactam antibiotics were classified into four classes, penams, cepheems, carbapenem and monobactam. One antibiotic name is listed in Figure 16 as an example under each class. The beta lactamase, which can open the beta lactam ring is indicated by R. For example, carbapenem is inactivated by KPC. To re-sensitise bacteria to carbapenem, the spacer sequence 5'-TTGTTGCTGAAGGAGTTGGG (SEQ ID NO: 45) should be employed into the spacer array and inactivate KPC genes. Note that the spacer sequence for CMY-a
25 can be employed to LAT-b cleavage.

Similarly, Figure 17 exemplifies a set of spacer sequences encoding 17 guide RNA molecules targeted against 154 different *bla* genes identified in the CARD database for *Klebsiella pneumoniae*. Beta lactamase gene type, spacer sequence and antibiotic
30 resistance profile in *Klebsiella pneumoniae* obtained from the IIDR CARD database are shown.

Beta lactamase gene sequences were collected by filtering all the collected beta lactamase genes with the keyword *Klebsiella pneumoniae* and subjected to multiple sequence
35 alignment using web program Clustal Omega. One canonical sequence from each cluster was chosen and the 20 nt spacer sequences predicted by the web program Jack Lin's CRISPR/Cas9 gRNA finder collected. The spacer sequence was chosen to maximise the

ratio of the proto-spacer sequence found in the sequences belonging to the same branch. The each of the example spacer sequences shown in the 4th row has the capability to disrupt the genes in the third column.

- 5 Beta lactamase genes used in this analysis are: SHV-a = 1a, 5, 2a, 11, 18, 20, 21, 22, 23, 28, 31, 32, 52, 55, 98, 99, 100, 106, 107, 110, 111, 121, 134, 136, 137, 140, 143, 144, 147, 148, 149, 150, 151, 152, 153, 154, 155, 157, 158, 159, 160, 161, 162, 163, 164, 165, 168, 172, 173, 178, 179, CTXM-b = 10, 12, 15, 52, 54, 62, 71, 80, CTXM-c = 19, 81, 99, 147, TEM-f = 1, 162, 183, 192, 197, 198, 209, KPC-g = 3, 4, 6, 7, 8, 11, 12, 14, 15, 16, 17, OKP-
10 h = 5, 6, A1, A2, A4, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, A15, A16, B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11, B12, B13, B17, B18, B19, B20, LEN-i = 5, 6, 7, 8, 9, 10, 11, 12, 13, 18, 19, 20, 21, 22, 23, 24, GES-j = 1, 3, 4, VIM-a = 4, 26, 27, 28, 33, 34, IMP-b = 32, 38, NDM-c = 1, 9, 10, ACT-3, CMY-a = 25, 31, 56, 87, FOX-d = 8, 9, OXA-a = 1, 30, 47, OXA-1, OXA-a = 181, 204, 247, OXA-9.

15

Beta lactam antibiotics are classified into four classes, penams, cepheems, carbapenem and monobactam. In Figure 17, one antibiotic name is listed as an example under each class. The beta lactamase, which can open the beta lactam ring is indicated by R. For example, carbapenem is inactivated by KPC. To re-sensitize bacteria to carbapenem, the spacer
20 sequence 5'-TTGTTGCTGAAGGAGTTGGG (SEQ ID NO: 45) should be employed into the spacer array and inactivate KPC genes.

Example 6

- 25 In this example, a modified Cas DNA-binding polypeptide is created that deletes and reseals rather than leaving a DSB.

As described above, DSBs caused by Cas DNA-binding polypeptide such as CRISPR-Cas9 can be lethal to the replicon targeted and can result in cell death rather than, for example, re-
30 sensitisation to antibiotics, if an antibiotic resistance gene in the replicon is targeted for inactivation. Immediate cell death rather than such re-sensitisation to antibiotics for subsequent killing by antibiotic may increase selection pressure against delivery of the targeting constructs. Instead of DSBs, we may modify the Cas9 gene to allow the resealing of the targeted sequence after gene inactivation by introducing a deletion or inversion.

35

Tsai et al. (2014) have constructed a fusion between a catalytically inactive Cas9 (dCas9) protein to the wild-type nuclease domain of the restriction endonuclease *FokI* to increase the

specificity of cleavage in eukaryotic cells. Proudfoot et al. (2011) have similarly fused zinc-finger DNA recognition domains to the catalytic domains of recombinases to programme site-specific recombination at designated DNA sequences.

- 5 We here add appropriate recombinase domains to dCas9 to catalyse a recombination reaction rather than a DSB at a CRISPR-mediated RNA-guided desired position on the target genes.

The Cas9 resolvase fusion, called Cas9R in Figure 18, is directed to the desired sites
 10 determined by the Cas9 domain:guide RNA spacer sequences; and the fused resolvase is positioned at the recombination site. To generate a deletion between two recombination sites: A and B, resolvases must dimerise at each recombination site. Thus two Cas9Rs need to be positioned in close proximity at each recombination site A1A2 and B1B2 as designated by the sequences encoded by the spacers S1-S4. The correct orientations of A1 relative to
 15 A2 and B1 relative to B2 will need to be determined experimentally.

The orientation of the gRNA as shown Fig. 19 is already proved in the case of dCas9 fused *FokI* (Tsai et al., 2014) - i.e. PAM sequences of each protospacer sequence in the recombination sites are directly positioned at the outer boundaries of the full-length
 20 recombination site. Thus, we employ the same configuration of the annealed gRNA polarity.

Figures 19 and 20 show a schematic process as to how this Cas9R fusion resolvase resolves the synapse and parental replicon DNA is recircularised.

25

Example 7

The following experiments describe some proof-of-concept experiments performed to demonstrate that the CRISPR-Cas9 system can be used in a single construct to inactivate
 30 a large number of different beta-lactamase genes that may be found amongst microbial pathogens as well as amongst the non-pathogenic members of the microbiome.

In these exemplifications, plasmids are constructed that carry the CRISPR-Cas9 system plus derivatives carrying spacer sequences, flanked by direct repeats, targeted against up
 35 to eight of the following beta-lactamase families of resistance genes: SHV, CTX-M, TEM, KPC, VIM, IMP, NDM and OXA.

For each of these eight families of beta-lactamase genes, a single spacer is designed that will target a number of gene members of that family. These are: SHV-a = 1, 1a, 2, 2a, 5, 5a, 11, 12, 14, 18, 20, 21, 22, 23, 26, 27, 28, 31, 32, 33, 38, 43, 44, 48, 52, 55, 56, 60, 61, 62, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 85, 89, 92, 98, 99, 100, 101, 103, 106, 107, 108, 109, 110, 111, 121, 136, 134, 137, 140, 143, 144, 147, 148, 149, 150, 151, 152, 153, 154, 155, 157, 158, 159, 160, 161, 162, 163, 164, 165, 168, 172, 173, 178, 179; CTXM-b= 1, 3, 10, 12, 15, 19, 22, 32, 52, 54, 59, 60, 62, 68, 71, 80, 81, 99, 141, 147; TEM-c = 1, 1B, 3, 139, 162, 183, 192, 197, 198, 209; KPC-d = 1, 2, 3, 4, 6, 7, 8, 11, 12, 14, 15, 16, 17 ; VIM-e = 1, 2, 4, 19, 26, 27, 33, 34; IMP-f = 4, 8, 32, 38; and NDM-g = 1, 9, 10.

10

Figure 21 shows the eight spacer sequences that were designed to target the eight beta-lactamase families of resistance genes: SHV, CTX-M, TEM, KPC, VIM, IMP, NDM and OXA-48. The primer sequences used in the construction of the plasmids are listed in a table in Figure 22.

15

In one exemplification, a plasmid derivative of pNB100 (Figure 12), pNB104A (Figure 23), a generally applicable DNA cassette, is described in the Examples that carries the CRISPR-Cas9 system plus derivatives carrying spacer sequences, flanked by direct repeats, targeted against four beta-lactamase families of antibiotic resistance genes in bacteria and are expressed off one promotor: NDM, IMP, VIM and KPC.

20

In another exemplification, a plasmid derivative of pNB100, pNB104B (Figure 24), a generally applicable DNA cassette, is described in the Examples that carries the CRISPR-Cas9 system plus derivatives carrying spacer sequences, flanked by direct repeats, targeted against four beta-lactamase families of antibiotic resistance genes in bacteria and are expressed off one promotor: OXA-48, SHV, TEM and CTX-M.

25

In another exemplification, a plasmid derivative of pNB100, pNB108 (Figure 25), a generally applicable DNA cassette, is described in the Examples that carries the CRISPR-Cas9 system plus derivatives carrying spacer sequences, flanked by direct repeats, targeted against eight beta-lactamase families of antibiotic resistance genes in bacteria and are expressed off one promotor: SHV, CTX-M, TEM, KPC, VIM, IMP, NDM and OXA-48.

30

In another exemplification, a plasmid derivative of pNB100 is constructed where spacer sequences can be expressed from a choice of two different promoters. This plasmid, pNB200 (Figure 26), carries a different unique restriction site downstream of each promoter to clone any desired spacer sequences flanked by their own direct repeats between two

35

direct repeats in the CRISPR locus. Derivatives of pNB200 are described that carry the CRISPR-Cas9 system plus derivatives carrying spacer sequences, flanked by direct repeats, targeted against eight beta-lactamase families of resistance genes: SHV, CTX-M, TEM, KPC, VIM, IMP, NDM and OXA-48. In pNB202 (Figure 27), the second promotor is
 5 used to express spacers flanked by direct repeats to target the four beta-lactamase families of resistance genes: OXA-48, SHV, TEM and CTX-M. And in pNB203 (Figure 28), which is derived from pNB202, both promotors are used: the first to express spacers flanked by direct repeats to target four beta-lactamase families of resistance genes: NDM, IMP, VIM and KPC and the second promotor to express spacers flanked by direct repeats to target
 10 four beta-lactamase families of resistance genes: OXA-48, SHV, TEM and CTX-M.

Construction of pNB104A

The tetramer spacer concatemer a+b+c+d shown in Figure 29A was digested with *BsaI*,
 15 whose restriction site is located in A1 and A2, and ligated to *BsaI* spacer cloning sites on pNB100. The structure of the single promoter and spacer region (6221-7001) on pNB104A is shown in Figure 23. The four-space concatemer contains spacer sequences targeting NDM, IMP, VIM and KPC from the proximal to the distal end of the single promoter.

20

Construction of pNB104B

The tetramer spacer sequence concatemer e+f+g+h shown in Figure 29A was digested with *SapI*, whose restriction site is located in B1 and B2, and ligated to *SapI* spacer cloning sites on pNB200. While screening pNB202 screening this construct was found and
 25 confirmed by sequencing. A deletion event between the direct repeats adjacent to leader sequences had occurred and restored the direct repeat sequence to give pNB104B. The single promoter regions (6221-6987) on pNB104B is shown in Figure 24. The concatenated spacers (targeted against OXA-48, SHV, TEM and CTX-M) are located under the single promoter.

30

Construction of pNB108

The concatenated spacer array sequences A and B were amplified from the subcloned vector pCR Blunt II-TOPO_SpacerA and pCR Blunt II-TOPO_SpacerB with the primer set
 35 NB026 and NB029, NB030 and NB033, respectively. At the 3' end of amplicon of spacer A and 5' end of amplicon of spacer B are 20 bases of overlapped sequence from KPC spacer sequence. These two amplicons were gel purified and used for PCR-based pairwise cycle

extension reaction in the absence of the primer. The extended material was re-amplified with primer set NB037 (5'-GGGCTGGCAAGCCACGTTTGGTG-3'; SEQ ID NO. 150) and NB038 (5'-CCGGGAGCTGCATGTGTCAGAGG-3'; SEQ ID NO. 151) to generate the full 8 spacer array concatemer. This eight-spacer concatemer was cloned into pCR Blunt II-TOPO vector and sequence was confirmed.

Bsal digestion of this pCR Blunt II-TOPO subclone removes the full 8 spacer array concatemer as a subclone from the pCR Blunt II-TOPO vector, which contains 5' protruding four base compatible bases on both ends for the sites created on pNB100 by *Bsal* digestion. Then pNB100 was digested with *Bsal* followed by agarose gel purification. The eight-spacer concatemer cassette, released from the pCR Blunt II-TOPO was ligated into pNB100 by T4 DNA ligase and transformed to DH5 α competent cells (purchased from New England Biolabs). The transformants were selected on chloramphenicol LB plates and were screened by PCR with the reverse primer NB021: 5'-GGTGAAGTATGGCCACGT (SEQ ID NO: 149) and a forward primer NB020: 5'-CCAACTACCTCCCCTTGCTTAAC (SEQ ID NO: 148), which anneal at 6368-6385 region and 7203-7225 region, respectively on the recombinant plasmid to generate 858 bp PCR amplicon. PCR-positive clones were sequenced to confirm the eight-spacer concatemer sequence and this recombinant clone is designated as pNB108 and used to demonstrate CRISPR/Cas9-mediated inactivation of targeted beta lactamase genes following DNA delivery to bacterial strains carrying such genes. A plasmid map of pNB108 is shown in Figure 25.

Construction of pNB200

The plasmid pNB200 contains two identical promoters for gRNA expression and carries a different unique restriction site *Bsal* and *SapI* downstream of each promoter to clone any desired spacer sequences flanked by their own direct repeats between two direct repeats in the CRISPR locus. The desired structure of the CRISPR array locus in pNB200 consists two sets of cassettes harbouring promoter-leader-direct repeat-spacer cloning region-direct repeat-tail tandemly. The forward primer NB018 anneals at the 5' end of the leader sequence to the promoter in pNB100 and introduces the first spacer cloning region, second direct repeat and tail sequence. The reverse primer NB019 anneals at the 3' end of leader sequence in pNB100 and introduces a third direct repeat, second spacer cloning region. This amplicon is cloned between *Bsal* sites on pNB100 to give pNB200.

35

The amplified sequence (SEQ ID NO: 146) with primer NB018 and NB019 from pNB100 as template

5' CCAAAActgagacctGCTGCGGACGTCCAAAGGCTCGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAACTTG
CCGATGATAACTTGAGAAAGAGGGTTAATACCAGCAGTCGGATACCTTCTATTCTTTCTGTTAAAGCGTTTTTCATGT
TATAATAGGCAAATTTTAGATGAAGATTATTTCTTAATAACTAAAAATATGGTATAATACTCTTAATAAATGCAGTAA
TACAGGGGCTTTTCAAGACTGAAGTCTAGCTGAGACAAATAGTGCGATTACGAAATTTTTAGACAAAAATAG ***TCTAC***
GAGGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAACTGAAGAGCGTCT ***CGGACgcagc***GCTCT ***TCGTTTTAGA***.

- 10 The forward primer NB018 and the reverse primer NB019 are underlined and the initial annealing sites, for the first PCR cycle, are indicated by bold letters and italicised bold letters, respectively. This amplicon was digested with *Bbv*I (5'-GCAGCN8/N12) to create four bases compatible protruding 5' ends to *Bsa*I digested pNB100. *Bbv*I recognition sites are depicted by lower bold cases.

- 15 The PCR conditions to generate the dual promotor-leader cassette were:

Component	
Nuclease-Free water	41.25 µL
10 X PCR Buffer	5 µL
10 mM dNTPs	1 µL
10 µM Forward Primer NB018	1 µL
10 µM Reverse Primer NB019	1 µL
pNB100	0.5 µL
QIAGEN Hot Start Taq	0.25 µL

- 20 Cycle conditions

STEP	TEMP	TIME
Initial Denat.	95 °C	15 min
35 Cycles	94 °C	30 sec
	55 °C	30 sec

	72 °C	30 sec
Final Extension	72 °C	10 min

The sequence of the modified CRISPR array in pNB200 (SEQ ID NO: 147)

5 The total number of nucleotides of pNB200 is 9919 bp. The sequence in the modified region is shown below, which replaces the small *Bsal* fragment in pNB100. The first and the second promoter sequences are underlined. Leader sequences are in bold case. Direct repeats are italicised and the spacer cloning region between direct repeats are indicated by italicised and underlined.

10

The first spacer array cloning region contains two inverted *Bsal* sites indicated by bold italicised underlined letters 5'-GAGACC-3' and 5'-GGTCTC-3' for creating 5' four bases protruding spacer cloning sites 5'-GTTT-3' and 5'-TTTT-3' on the vector, respectively and one unique *AatII* (5'-GACGTC-3') site also indicated by bold italicised underlined letters to

15 reduce self-ligation in the event of incomplete *Bsal* digestion.

The second spacer cloning region contains two inverted *SapI* sites indicated by bold italicised underlined letters 5'-GAAGAGC-3' and 5'-GCTCTTC-3' for creating 5' three bases protruding spacer cloning sites 5'-GTT-3' and 5'-TTT-3' on the vector, respectively.

20

GGTGACTGATGGCCACGTGAACCTATATGATTTTCCGCAGTATATTTTAGATGAAGATTATTTCTTAATAACTAAAAAT
 ATGGTATAATACTCTT**AATAAATGCAGTAATACAGGGGCTTTTCAAGACTGAAGTCTAGCTGAGACAAATAGTGCGAT**
TACGAAATTTTTTAGACAAAAATAGTCTACGAGGTTTAGAGCTATGCTATTTTGAATGGTCCCAAAAC**tGAGACCTG**
 25 **CTGCGGACGTC**CAAA**GGTCTC**GTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACttgccgatgataaacttgagaa
 agaggggttaataaccagcagtcggataccttcctattctcttctgttaaagcggtttcatgttataataggcaa**ATTTTA**
GATGAAGATTATTTCTTAATAACTAAAAATATGGTATAATACTCTT**AATAAATGCAGTAATACAGGGGCTTTCAAGA**
CTGAAGTCTAGCTGAGACAAATAGTGCGATTACGAAATTTTTTAGACAAAAATAGTCTACGAGGTTTTAGAGCTATGC
TGTTTTGAATGGTCCCAAAAC**tGAAGAGCTCTCGGACGCAGCGCTCTTC**GTTTTAGAGCTATGCTGTTTTGAATGGT
 30 **CCCAAAAC**aacattgccgatgataaacttgagaaagaggggttaataaccagcagtcggataccttcctattcttctgtt
 aaagcggtttcatgttataataggcaaaagaagagtagtg

A plasmid map of pNB200 is shown in Figure 26. The desired spacer array sequences can be cloned in the clockwise direction between two *Bsal* sites and two *SapI* sites
 35 independently, for each promoter respectively.

Construction of pNB202

The plasmid pNB201 contains the dual promoters of pNB200 from which it is derived but a
 5 spacer array sequence targeted to OXA-48, SHV, TEM and CTX-M beta lactamase gene
 families are expressed from the second promoter.

To construct pNB202, pNB200 was digested with *Bsal* followed by agarose gel purification.
 The concatenated spacer array sequence B was generated by PCR-mediated pair-wise
 10 oligo nucleotide concatenation. This four-spacer concatemer array B was cloned to the
 pCR Blunt II-TOPO vector, purchased from Life Technologies, and the sequence was
 confirmed. *SapI* cuts out spacer array sequence B as a subclone from the TOPO vector
 and yields 5' protruding three base compatible bases on both ends for the sites created on
 pNB200 by *SapI* digestion. This four-spacer concatemer B cassette was ligated to pNB200
 15 by T4 DNA ligase and transformed to DH5α competent cells (purchased from New England
 Biolabs). The transformants were selected on chloramphenicol LB plate and were screened
 by PCR with the reverse primer NB021: 5'- GGTGACTGATGGCCACGT (SEQ ID NO: 149)
 and a forward primer NB020: 5'- CCAACTACCTCCCCTTGCTTAAC (SEQ ID NO: 148),
 which anneal at 6368-6385 region and 7307-7329 region, respectively on the recombinant
 20 plasmid to generate 962 bp PCR amplicon. PCR positive clones were sequenced to
 confirm the four-spacer concatemer B sequence and this recombinant clone is designated
 as pNB202 and used to demonstrate CRISPR/Cas9-mediated inactivation of targeted beta
 lactamase genes following DNA delivery to bacterial strains carrying such genes. A plasmid
 map of pNB202 is shown in Figure 27.

25

Construction of pNB203

The plasmid pNB203 contains the dual promoters of pNB200 but each promoter expresses
 four spacers. The first promoter expresses spacer sequence targeted to NDM, IMP, VIM
 30 and KPC, the second promoter expresses OXA, SHV, TEM and CTX-M beta lactamase
 genes. The plasmid pNB202 was digested with *Bsal* followed by agarose gel purification.
 The concatenated spacer array sequence A was cut out from pCR Blunt II-TOPO vector
 harbouring spacer concatemer A with *Bsal*. *Bsal* cuts out spacer region, which contains 5'
 protruding four base compatible bases on both ends for the sites created on pNB202 by
 35 *Bsal* digestion. This four-spacer concatemer A cassette was ligated to pNB202 by T4 DNA
 ligase and transformed to DH5α competent cells (purchased from New England Biolabs).
 The transformants were selected on chloramphenicol LB plate and were screened by PCR

with the reverse primer NB021: 5'- GGTGACTGATGGCCACGT and a forward primer NB020: 5'- CCAACTACCTCCCCTTGCTTAAC , which anneal at 6368-6385 region and 7479-7501 region, respectively on the recombinant plasmid to generate 1134 bp PCR amplicon. PCR positive clones were sequenced to confirm the four-spacer concatemer A and B sequence and this recombinant clone is designated as pNB203 and used to demonstrate CRISPR/Cas9-mediated inactivation of targeted beta lactamase genes following DNA delivery to bacterial strains carrying such genes. A plasmid map of pNB203 is shown in Figure 28.

10

Schematic representation of the structure of concatenated spacer arrays

Spacer sequences were determined to maximise the coverage of the target beta-lactamase gene family. Each unit oligo contains the direct repeat flanking the appropriate spacer sequence at each end. Concatenation reactions are performed between pairwise oligos, *i.e.* the nearest neighbour unit oligos are concatenated first to generate two unit length oligo, then two unit length oligos are concatenated to generate four unit length of oligo etc.

15

The schematic structure of tetramer and octamer spacer structures are shown in Figure 29. S: spacer, R: direct repeat, A and B contain *BsaI* site to create ligation compatible sites for cloning into pNB100 and downstream of the first promotor of pNB200. C and D contain a *SapI* site to create ligation a compatible site for cloning downstream of the second promotor of pNB200. In this example, we employed spacer sequence S1 targeting NDM, S2 targeting IMP, S3 targeting VIM, S4 targeting KPC, S5 targeting OXA, S6 targeting SHV, S7 targeting TEM and S8 targeting CTX-M.

20

Spacer concatenation reaction

Each oligo has overlapped sequence in the 3' and 5' end to anneal to the nearest neighbour oligo except the first and the last oligo. The first and the last oligo have the overlapping sequence to the second and the penultimate oligo in the 5' end only. In order to concatenate four spacers, four oligos are synthesised. In other words, oligo No.1 consists spacer 1 and 2 in the 5' and 3' ends. Oligo No.2 contains reverse complement of spacer 2 and 3 in the 3' and 5' ends. Oligo No.3 contains spacer 3 and 4 in the 5' and 3' ends. Oligo No.4 contains reverse complement of spacer 4 in the 3' end. Thus the oligo No.2 can link oligo No.1 and oligo No.3, oligo 4 anneals to 3' end of oligo No.3. Oligo No.1 and oligo No. 2, oligo No. 3 and oligo No. 4 are concatenated in a separate tube using the following PCR reaction conditions.

30

35

Component	A1	A2	B1	B2	x 4
Nuclease-Free water	33.5 μ L	33.5 μ L	33.5 μ L	33.5 μ L	134
5X Q5 Reaction Buffer	10 μ L	10 μ L	10 μ L	10 μ L	40
10 mM dNTPs	1 μ L	1 μ L	1 μ L	1 μ L	4
Q5 High-Fidelity DNA Polymerase	0.5 μ L	0.5 μ L	0.5 μ L	0.5 μ L	2
10 μ M Forward Primer NB026	2.5 μ L				
10 μ M Reverse Primer NB027	2.5 μ L				
10 μ M Forward Primer NB028		2.5 μ L			
10 μ M Reverse Primer NB034		2.5 μ L			
10 μ M Forward Primer NB035			2.5 μ L		
10 μ M Reverse Primer NB031			2.5 μ L		
10 μ M Forward Primer NB032				2.5 μ L	
10 μ M Reverse Primer NB036				2.5 μ L	

Cycle conditions

STEP	TEMP	TIME
Initial Denat.	98 °C	60 sec
35 cycles	98 °C	10 sec
	55 °C	10 sec
	72 °C	20 sec
Final Extension	72 °C	2 minutes
Hold	4 °C	

- 5 In this example, NB026 and NB027, NB028 and NB034, NB035 and NB031, NB032 and NB036 are concatenated. Each concatenated product A1, A2, B1 and B2 was gel purified

and set up the second concatenation reaction using the purified A1 and A2, B1 and B2 dimer product in the following PCR condition.

Component	A	B	x 2
Nuclease-Free water	35.75 μ L	35.75 μ L	71.5
10 X PCR Buffer	5 μ L	5 μ L	10
10 mM dNTPs	1 μ L	1 μ L	2
QIAGEN Hot Start Taq	0.25 μ L	0.25 μ L	0.5
Gel extracted A1	4 μ L		
Gel extracted A2	4 μ L		
Gel extracted B1		4 μ L	
Gel extracted B2		4 μ L	

5 Cycle conditions

STEP	TEMP	TIME
Initial Denat.	95°C	15 min
35 Cycles	94°C	30 sec
	A: 55°C	30 sec
	72°C	30 sec
Final Extension	72°C	10 min

These extension products were amplified by NB037 and NB038 with Q5 DNA polymerase.

The final amplicons were cloned to pCR Blunt II TOPO vector and the concatemer

10 sequences were confirmed.

In case of eight spacer concatenation, spacer concatemer A and spacer concatemer B on pCR Blunt II TOPO vector were amplified with primer pairs NB026 and NB029, NB030 and NB033, respectively and amplicons were gel purified. Purified spacer A and B were utilised as a long primer in the following cycle extension reaction.

5

Component	A
Nuclease-Free water	35.75 μ L
10 X PCR Buffer	5 μ L
10 mM dNTPs	1 μ L
QIAGEN Hot Start Taq	0.25 μ L
Gel extracted A	4 μ L
Gel extracted B	4 μ L

Cycle conditions

STEP	TEMP	TIME
Initial Denat.	95°C	15 min
25 Cycles	94°C	30 sec
	55°C	30 sec
	72°C	30 sec
Final Extension	72°C	10 min
Hold	4 °C	o/n

- 10 These extension products were amplified by NB037 and NB038 with Q5 DNA polymerase. The final amplicons were cloned into pCR Blunt II TOPO vector and the concatemer sequences were confirmed.

Delivery of CRISPR-Cas9 constructs to bacteria

Constructs that are able to inactivate target genes, including antibiotic resistant genes, via the CRISPR-Cas system, and which are an aspect of the present invention are also referred to herein as “Nemesis symbiotics”. The CRISPR-Cas9 plasmid derivatives, pNB104A, pNB104B, pNB202 and pNB203 all carry spacer insertions targeted against the selected families of beta-lactamase (*bla*) genes described, and so provide exemplars to demonstrate that a single plasmid construct possesses Nemesis symbiotic activity (NSA) and is therefore able to inactivate representative genes from all 8 different families of beta-lactam antibiotics. The plasmid pNB104A (see Fig. 23) is tested for its ability to inactivate representative genes members of the NDM, IMP, VIM and KPC families; pNB104B (see Fig. 24) and pNB202 (ss Fig. 27) are tested for their ability to inactivate representative genes members of the OXA, SHV, TEM and CTX-M families; and pNB108 (see Fig. 25) and pNB203 (see Fig. 28) are tested for their ability to inactivate representative genes members of the SHV, CTX-M, TEM, KPC, VIM, IMP, NDM and OXA families.

Nemesis symbiotic activity (NSA) assay by plasmid transformation

The NSA assay described in Example 2 showed that DNA transformation of an *E. coli* strain, DH5 α , also carrying the TEM-3 beta lactamase gene on the plasmid pBR322, with plasmid pNB102 converts the transformant to ampicillin sensitivity (ApS). Here, the plasmid pNB102 encodes resistance to chloramphenicol and the DH5 α (pBR322) transformants now carrying pNB102 were selected on LB Cm plates and then screened for ApS (see Fig. 14). Here, the plasmid pNB102, in expressing the CRISPR/Cas9 system with the spacer sequence encoding the gRNA targeting the TEM-3 gene, inactivated the TEM-3 gene. In contrast in a negative control experiment, when DH5 α (pBR322) was transformed with the parental plasmid pNB100 carrying the expressing the CRISPR/Cas9 system but lacking the gRNA targeting the TEM-3 gene, no conversion to ApS occurred.

For exemplification purposes an equivalent experiment is described where plasmid derivatives of pBR322 are constructed where the TEM-3 is replaced by representative genes from the other 7 different families of beta-lactam antibiotics: SHV, CTX-M, KPC, VIM, IMP, NDM and OXA. Such genes are obtained from suitable bacterial strains carrying such genes. This allows a direct comparison to the proof of concept experiments described in Example 2 in isogenic genetic backgrounds.

A set of *E. coli* and *K. pneumoniae* strains carrying representative genes from these seven different families of beta-lactam antibiotics were purchased from Culture Collections, Public

Health England, Porton Down, Salisbury, SP4 0JG, UK. These are: NCTC13368, a *K. pneumoniae* strain carrying the SHV-18 gene; NCTC13353 an *E. coli* strain carrying the CTX-M-15 gene; NCTC13438 a *K. pneumoniae* strain carrying the KPC-3 gene; NCTC13440 a *K. pneumoniae* strain carrying the VIM-1 gene; NCTC13476 an *E. coli* strain carrying an uncharacterised IMP gene; NCTC13443 a *K. pneumoniae* strain carrying the NDM-1 gene and NCTC13442 a *K. pneumoniae* strain carrying the OXA-48 gene. All seven genes encode beta lactamases that are also able to degrade and inactivate the penam class of antibiotics (see Fig. 16, 17). All strains were tested and, as expected, found to be resistant to the penam class antibiotic, ampicillin.

10

Beta lactamase coding sequences are amplified from the cell with appropriate forward and reverse primer set shown below:

Strain	NCTC No.	Resistance gene	Forward primer 5' to 3'	Reverse primer 5' to 3'
NBKp001	13443	NDM-1	attgaaaaaggaagagtATGGAATTGCCC AATATTATGCACCC (SEQ ID NO: 152)	agtcccgctaGGTCTCaACCGTCAGCGCA GCTTGTCGG (SEQ ID NO: 153)
NBKp002	13442	OXA-48	attgaaaaaggaagagtATGCGTGATTA GCCTTATCGGCTG (SEQ ID NO: 154)	agtcccgctaGGTCTCaACCGCTAGGGAA TAATTTTTCCTGTTTGAGCACTTCT (SEQ ID NO: 155)
NBKp003	13368	SHV-18	attgaaaaaggaagagtATGCGTTATTTT CGCCTGTGTATTATCTCC (SEQ ID NO: 156)	agtcccgctaGGTCTCaACCGTTAGCGTT GCCAGTGCTCGA (SEQ ID NO: 157)
NBKp004	13440	VIM-1	attgaaaaaggaagagtATGTTAAAAGTT ATTAGTAGTTTATTGGTCTACATGACCG (SEQ ID NO: 158)	agtcccgctaGGATGacctggtgACCGC TACTCGGCGACTGAGCGAT (SEQ ID NO: 159)
NBKp005	13438	KPC-3	attgaaaaaggaagagtATGTCACGTGTAT CGCCGTCTAGTTCT (SEQ ID NO: 160)	agtcccgctaGGTCTCaACCGTTACTGCC CGTTGACGCC (SEQ ID NO: 161)
NBec018	13476	IMP-4	attgaaaaaggaagagtATGAGCAAGTTA TCTGTATTCTTTATATTTTGTGTTAG CA (SEQ ID NO: 162)	agtcccgctaGGTCTCaACCGTTAGTTGC TTAGTTTGTATGTTTTTACTTTCGTT AAC (SEQ ID NO: 163)
NBec019	13353	CTX-M-15	attgaaaaaggaagagtATGGTTAAAAA TCACTGCGCCAGTTC (SEQ ID NO: 164)	agtcccgctaGGTCTCaACCGTTACAAAC CGTCGGTGACGATTTTAG (SEQ ID NO: 165)

15

Each forward primer contains a 17 base sequence to restore the beta-lactamase promoter on pBR322, and each reverse primer contains *Bsa*I site (for NDM-1, OXA-48, SHV-18, KPC-3, IMP-4 and CTX-M15) or *Fok*I site (for VIM-1) to create 5'-ACCG four base protruding 5' end. After amplifying each beta lactamase gene with high fidelity DNA polymerase such as Q5 DNA polymerase, the amplicon is digested with the appropriate restriction enzyme located in the reverse primer, described above. The digested amplicons are ready to ligate using T4 ligase between the *Ssp*I and *Bsa*I sites on the plasmid pBR322 (purchased from New England Biolabs), after removal of the TEM-3 fragment. *Ssp*I creates a blunt end and *Bsa*I creates a 5'-CGGT protruding end. The reverse complement of the

20

coding sequences of the each amplicons after restriction digestion are shown below. The 5' protruding end is underlined and 3' end of the promotor sequence is in bold small letters.

The reverse complement CAT of the methionine initiating codons ATG of these seven genes, also shown in bold, yields a precise fusion of the coding region of the seven other

- 5 beta-lactamases to the translational signal sequences of the TEM-3 beta-lactamase of pBR322.

NDM-1 (SEQ ID NO: 166)

10 ACCGTCAGCGCAGCTTGTTCGGCCATGCGGGCCGTATGAGTGATTGCGGCGCGGCTATCGGGGGCGGA
 ATGGCTCATCACGATCATGCTGGCCTTGGGGAACGCCGACCAAACGCGCGCGCTGACGCGGCGTAG
 TGCTCAGTGTTCGGCATCACCGAGATTGCCGAGCGACTTGGCCTTGCTGTCCTTGATCAGGCAGCCAC
 CAAAAGCGATGTTCGGTGGCGTCGATCCCAACGGTGATATTGTCACTGGTGTGGCCGGGGCCGGGGTA
 AAATACCTTGAGCGGGCCAAAGTTGGGCGCGGTTGCTGGTTCGACCCAGCCATTGGCGGCGAAAAGTC
 15 AGGCTGTGTTGCGCCGCAACCATCCCCCTCTTGGGGGCAAGCTGGTTCGACAACGCATTGGCATAAG
 TCGCAATCCCCGCCGATGCAGCGCGTCCATACCGCCCATCTTGTCTGATGCGCGTGAGTCACCAC
 CGCCAGCGCGACCGGCAGGTTGATCTCCTGCTTGATCCAGTTGAGGATCTGGGCGGCTCTGGTCATCG
 GTCCAGGCGGTATCGACCACCAGCACGCGGCGCCATCCCTGACGATCAAACCGTTGGAAGCGACTG
 CCCCAGAAACCCGGCATGTCGAGATAGGAAGTGTGCTGCCAGACATTTCGGTTCGAGCTGGCGGAAAAC
 20 CAGATCGCCAAACCGTTGGTCGCCAGTTTCCATTTGCTGGCCAATCGTCGGGCGGATTTACCGGGC
 ATGCACCCGCTCAGCATCAATGCAGCGGCTAATGCGGTGCTCAGCTTCGCGACCGGGTGCATAATAT
 TGGGCAATT**CATactcttctttttcaat**

OXA-48 (SEQ ID NO: 167)

25 ACCGCTAGGGAATAATTTTTTCTGTTTGAGCACTTCTTTTTGTGATGGCTTGGCGCAGCCCTAAACC
 ATCCGATGTGGGCATATCCATATTCATCGCAAAAACACACATTATCATCAAGTTCAACCCAAACCG
 ACCCACCAGCCAATCTTAGGTTTCGATTCTAGTCGAGTATCCAGTTTTAGCCCGAATAATATAGTCAC
 CATTGGCTTCGGTCAGCATGGCTTGTTTGACAATACGCTGGCTGCGCTCCGATACGTGTAACCTTATT
 30 GTGATACAGCTTTCTTAAAAAGCTGATTTGCTCCGTGGCCGAAATTCGAATACCACCGTCGAGCCAG
 AAAGTGTCTACATTGCCCGAAATGTCCTCATTACCATAATCGAAAGCATGTAGCATCTTGCTCATAAC
 GTGCCTCGCCAATTTGGCGGGCAAATTTCTTGATAAACAGGCACAACCTGAATATTTTCATCGCGGTGAT
 TAGATTATGATCGCGATTCCAAGTGGCGATATCGCGCGTCTGTCCATCCCACTTAAAGACTTGGTGT
 TCATCCTTAACACGCCCAAATCGAGGGCGATCAAGCTATTGGGAATTTTAAAGGTAGATGCGGGTA
 35 AAAATGCTTGGTTCGCCCGTTTAAAGATTATTGGTAAATCCTTGCTGCTTATTCTCATTCCAGAGCAC
 AACTACGCCCTGTGATTTATGTTTCAGTAAAGTGAGCATTTCCAACCTTTGTTTTCTTGCCATTCCCTT
 GCTACCGCAGGCATTCCGATAATCGATGCCACCAAAAACACAGCCGATAAGGCTAATACACG**CATac**
tcttctttttcaat

40 SHV-18 (SEQ ID NO: 168)

ACCGTTAGCGTTGCCAGTGCTCGATCAGCGCCGCGCCGATCCCGGCGATTTGCTGATTTGCTCGGC
 CATGCTCGCCGGCGTATCCCGCAGATAAATCACCACAATCCGCTCTGCTTTGTTATTCGGGCCAAGC
 AGGGCGACAATCCCGCGCGCACCCCGTTTGGCAGCTCCGGTCTTATCGGCGATAAACAGCCCGCCG
 45 GCAGCACGGAGCGGATCAACGGTCCGGCGACCCGATCGTCCACCATCCACTGCAGCAGCTGCCGTTG
 CGAACGGGCGCTCAGACGCTGGCTGGTCAGCAGCTTGCGCAGGGTCGCGGCCATGCTGGCCGGGGTA
 GTGGTGTGCGGGCGTTCGCCGGGAAGCGCCTCATTAGTTCCGTTTCCCAGCGGTCAAGGCGGGTGA
 CGTTGTGCGCGATCTGGCGCAAAAAGGCAGTCAATCCTGCGGGGCCGCGACGGTGGCCAGCAGCAG
 ATTGGCGGCGCTGTTATCGCTCATGGTAATGGCGGCGGCACAGAGTTCGCCGACCGTCATGCCGTG
 50 GCAAGGTGTTTTTCGCTGACCGGCGAGTAGTCCACCAGATCCTGCTGGCGATAGTGATCTTTTCGCT
 CCAGCTGTTTCGTCACCGGCATCCACCCGCGCCAGCACTGCGCCGAGAGCACTACTTTAAAGGTGCT
 CATCATGGGAAAGCGTTTCATCGGCGCGCCAGGCGGTGAGCGTGCAGGCGGCTGGCCAGATCCATTTCT
 ATCATGCCCTACGCTGCCGACAGCTGGCTTTCGCTTAGTTTAATTTGCTCAAGCGGCTGCGGGCTGG
 CGTGTACCGCCAGGCGAGGGTGGCTAACAGGGAGATAATACACAGGCGAAAATAACG**CATactctt**
 55 **cctttttcaat**

VIM-1 (SEQ ID NO: 169)

ACCGCTACTCGGCGACTGAGCGATTTTTGTGTGCTTTGACAACGTTTCGCTGTGTGCTGGAGCAAGTC
 TAGACCGCCCGGTAGACCGTGCCCGGGAATGACGACCTCTGCTTCCGGGTAGTGTTTTTGAATCCGC
 TCAACGGAGGTGGGCCATTACAGCCAGATCGGCATCGGCCACGTTCCCCGCAGACGTGCTTGACAACT
 5 CATGAACGGGCACAACCACCGTATAGCACGTTTCGCTGACGGGACGTATACAACCAGATTGTTCGGTCGA
 ATGCGCAGCACCAAGGATAGAAGAGCTCTACTGGACCGAAGCGCACTGCGTCCCCGCTCGATGAGAGT
 CTTTCTAGAGAAATGCGTGGGAATCTCGTTCCCCCTCTGCCTCGGCTAGCCGGCGTGTGACGGTGATG
 CGTACGTTGCCACCCAGCCGCCGAAGGACATCAACGCCGCCGACGCGGTGCTCATGAAAGTGCCT
 GGAGACTGCACGCGTTACGGGAAGTCCAATTTGCTTTTCAATCTCCGCGAGAAGTGCCGCTGTGTTT
 10 TTCGCACCCACGCTGTATCAATCAAAAGCAACTCATCACCATCACGGACAATGAGACCATTGGACG
 GGTAGACCGCGCCATCAAACGACTGCGTTGCGATATGCGACCAAACACCATCGGCAATCTGGTAAAG
 TCGGACCTCTCCGACCGGAATTTGCTTGACTGTCGGATACTCACCCTCGGCTCCCCGGAATGGGCT
 AACGGAATTGCGACAGCCATGACAGACGCGGTTCATGTAGACCAATAAACTACTAATAACTTTTAA**CA**
Tactcttcctttttcaat

15
 KPC-3 (SEQ ID NO: 170)

ACCGTTACTGCCCCGTTGACGCCCAATCCCTCGAGCGCGAGTCTAGCCGCAGCGGCGATGACGGCCTC
 GCTGTACTTTGTCATCCTTGTTAGGCGCCCGGGTGTAGACGGCCAACACAATAGGTGCGCGCCAGTG
 20 GGCCAGACGACGGCATAGTCATTTGCCGTGCCATACACTCCGCAGGTTCGGGTTTTGTCTCCGACTG
 CCCAGTCTGCCGGCACCGCCGCGCGGATGCGGTGGTTGCCGGTTCGTGTTTCCCTTTAGCCAATCAAC
 AAATGCTGCGCGTGC GGCGCAGCCAGTGCAGAGCCCAGTGTGAGTTTTGTAAGCTTTCCGTCACG
 GCGCGCGGCGATGAGGTATCGCGCGCATCGCCTGGGATGGCGGAGTTCAGCTCCAGCTCCCAGCGGT
 CCAGACGGAACGTGGTATCGCCGATAGAGCGCATGAAGGCCGTCAGCCCGGGCCGCCCAACTC
 25 CTTAGCAACAAATTGGCGGCGGCGTTATCACTGTATTGCACGGCGGCGCGGACAGCTCCGCCACC
 GTCATGCCTGTTGTCAGATATTTTTCCGAGATGGGTGACCACGGAACCAGCGCATTTTTTGCCGTAAC
 GGATGGGTGTGTCCAGCAAGCCGGCCTGCTGCTGGCTGCGAGCCAGCACAGCGGCAGCAAGAAAGCC
 CTTGAATGAGCTGCACAGTGGGAAGCGCTCCTCAGCGCGGTAACCTTACAGTTGCGCCTGAGCCGGTA
 TCCATCGCGTACACACCGATGGAGCCGCCAAAGTCCTGTTTCGAGTTTAGCGAATGGTTCCGCGACGA
 30 GGTGGTTCAGCGCGGTGGCAGAAAAGCCAGCCAGCGGCCATGAGAGACAAGACAGCAGAACTAGACG
 GCGATACAGTGA**CA****Tactcttcctttttcaat**

IMP-4 (SEQ ID NO: 171)

ACCGTTAGTTGCTTAGTTTTGATGGTTTTTTACTTTTCGTTTAAACCCTTTAACC GCCTGCTCTAATGT
 AAGTTTTCAAGAGTGATGCGTCTCCAGCTTCACTGTGACTTGGAACAACCAGTTTTGCCTTACCATAT
 TTGGATATTAATAATTTAGCGGACTTTGGCCAAGCTTCTAAATTTGCGTCACCCAAATTACCTAGAC
 CGTACGGTTTTAATAAAACAACCACCGAATAATATTTTCCTTTTCAGGCAGCCAACTACTAGGTTATC
 TGGAGTGTGCTTGGGCTGGATAAAAAACTTCAATTTTTATTTTTAACTAGCCAATAGTTAACCCCG
 40 CCAATGAATTTTTAGCTTGAACCTTACCGTCTTTTTTAAAGCAGCTCATTAGTTAATTAGACGCAT
 ACGTGGGGATGGATTGAGAATTAAGCCACTCTATTCGCCCGTGTGCTACTATGAAAATGAGAGGA
 AATACTGCCTTTTATTTTATAGCCACGTTCCACAAACCAAGTGACTAATTTTCAGTATCTTTAGCC
 GTAAATGGAGTGTCAATTAGATAAGCTTCAGCATCTACAAGAACAACCAACCATGTTTAGGAACAA
 CGCCCCACCCGTTAACTTCTTCAAACGAAGTATGAACATAAACGCCTTCATCAAGTTTTTTCAATTTT
 45 TAAATCTGGCAAAGACTCTGCTGCGGTAGCAATGCTACAAAACAAAATATAAAGAATACAGATAAC
 TTGCT**CA****Tactcttcctttttcaat**

CTX-M-15 (SEQ ID NO: 172)

ACCGTTACAAACCGTCGGTGACGATTTTAGCCGCCGACGCTAATACATCGCGACGGCTTTCTGCCTT
 AGGTTGAGGCTGGGTGAAGTAAGTGACCAGAATCAGCGGCGCACGATCTTTTGCCAGATCACCGCG
 ATATCGTTGGTGGTGCCATAGCCACCGCTGCCGTTTTATCCCCCACAACCCAGGAAGCAGGCAGTC
 CAGCCTGAATGCTCGCTGCACCGGTGGTATTGCCTTTCATCCATGTCACCAGCTGCGCCCGTTGGCT
 GTCGCCCAATGCTTTACCCAGCGTCAGATTCCGCAGAGTTTGCGCCATTGCCCGAGGTGAAGTGGTA
 55 TCACGCGGATCGCCCGAATGGCGGTGTTAACGTCGGCTCGGTACGGTCGAGACGGAACGTTTCGT
 CTCCAGCTGTGCGGCGAACGCGGTGACGCTAGCCGGGCCGCCAACGTGAGCAATCAGCTTATTCAT
 CGCCACGTTATCGCTGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCAGTGACATCGTCCCATTGACG
 TGCTTTTCCGCAATCGGATTATAGTTAAACAAGGTCAGATTTTTTGATCTCAACTCGCTGATTTAACA
 GATTCCGTTTCGCTTTCACTTTTCTTACGACCGCGGCCGCGGCCATCACTTTACTGGTGTGCACAT
 60 GCCTAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCTGTGTTAATCAATGCCACACC
 AGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGCCGTTTGCGCATACA

GCGGCACACTTCCTAACAACAGCGTGACGGTTGCCGTCGCCATCAGCGTGAAGTGGCGCAGTGATT
TTTAACCATactcttcctttttcaat

- 5 Then DH5α competent cells purchased from New England Biolabs are transformed with these ligations followed by selection for the desired recombinants on LB Ampicillin (100μg/mL) plates.

Plasmid DNA samples are isolated from these transformants and submitted to DNA

- 10 sequence analysis to confirm that the correct sequence for each of the seven different beta lactamases genes is present in each construct giving the plasmids:

These pBR322 derivative plasmids so derived are named:

- i. pNB010 carrying the SHV-18 gene;
- 15 ii. pNB011 carrying the CTX-M-15 gene;
- iii. pNB012 carrying the KPC-3 gene;
- iv. pNB013 carrying the VIM-1 gene;
- v. pNB014 carrying the IMP gene;
- vi. pNB015 carrying the NDM-1 gene;
- 20 vii. pNB016 carrying the OXA-48 gene; in addition to, as described,
- viii. pBR322 carrying the TEM-3 gene

Then 7 recipient *E. coli* strains, DH5α, each carrying one of these seven beta lactamase genes on the plasmids pNB010-016 are subsequently transformed with the plasmids

- 25 pNB104A, pNB104B, pNB108 and pNB203 and selected for chloramphenicol resistance to select for acquisition of these Nemesis symbiotic plasmids, along with the negative control pNB100 as well as with transformation of DH5α (pBR322) by pNB102 as the positive control, and then tested for conversion to ampicillin sensitivity as described in Example 2 (see Fig 14.)

30

“Nemesis symbiotic activity” (NSA) assay by plasmid conjugation to test activity of constructs carrying multiple spacers targeting beta lactamase gene families

The plasmid conjugation assay described in Example 2 may also be used to test the Nemesis symbiotic activity of the new CRISPR/Cas9 plasmid constructs carrying spacer

- 35 sequences targeting multiple families of beta lactamase genes. The assay involves mating a donor cell carrying the beta lactamase gene on a conjugative plasmid, and hence ampicillin resistant, with a recipient cell carrying the Nemesis symbiotic on a non-

mobilisable plasmid encoding chloramphenicol resistance. Exconjugants are selected on LB plates containing both 100µg/mL ampicillin and 35µg/mL chloramphenicol. Successful Nemesis symbiotic activity is seen in a reduction in the efficiency of transfer of the ampicillin resistance gene.

5

As donors, the same strain used in Example 2 was used. This strain, JA200 (F+ thr-1, leu-6, DE(trpE)5, recA, lacY, thi, gal, xyl, ara, mtl), also carries plasmid pNT3 as described by Saka et al. (DNA Research 12, 63–68, 2005). The plasmid pNT3 is a mobilisable plasmid carrying the TEM-1 beta lactamase gene of Tn1. Conjugation of pNT3 and hence transfer
10 of ampicillin resistance is effected by the transfer functions of the co-resident F+ plasmid.

The other potential donors are the set of *E. coli* and *K. pneumoniae* strains carrying representative genes from these seven different families of beta-lactam antibiotics that were purchased from Culture Collections, Public Health England, as described above. These strains need to be chloramphenicol sensitive in order to allow selection for
15 exconjugants and were tested for growth on LB chloramphenicol 35µg/mL plates. NCTC13368, a *K. pneumoniae* strain carrying the SHV-18 gene; NCTC13438 a *K. pneumoniae* strain carrying the KPC-3 gene; NCTC13476 an *E. coli* strain carrying an uncharacterised IMP gene; NCTC13443 a *K. pneumoniae* strain carrying the NDM-1 gene and NCTC13442 a *K. pneumoniae* strain carrying the OXA-48 gene were all found to be
20 resistant to chloramphenicol, but NCTC13353, an *E. coli* strain carrying the CTX-M-15 gene, and NCTC13440, a *K. pneumoniae* strain carrying the VIM-1 gene, were found to be chloramphenicol sensitive and were taken forward to be tested in matings with the recipients.

As recipients, the strains used in Example 2 serve as controls for testing the new plasmid
25 constructs. Thus the negative control is DH5α (F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ with the plasmid pNB100 encoding the CRISPR/Cas9 cassette but no spacer sequence targeting any antibiotic resistance gene; and the positive control is DH5α with the plasmid pNB102 encoding the CRISPR/Cas9 cassette as well as the spacer sequence targeting the TEM
30 beta-lactamase family. To be tested are DH5α strains with the plasmids pNB104A, pNB104B and pNB108. These plasmids also encode the CRISPR/Cas9 cassette in addition to the spacer sequences, all driven off one promotor in the following order, proximal to distal from the promotor (pNB104A): NDM–IMP–VIM–KPC; (pNB104B): OXA-48–SHV–TEM–CTX-M (pNB108): NDM–IMP–VIM–KPC–OXA-48–SHV–TEM–CTX-M.

35

To set up the matings, colonies of donors were picked from LB Ap100 (ampicillin 100µg/mL) and recipients from LB Cm35 (chloramphenicol 35µg/mL) plates into 200µL of LB and then mixed 100µL of recipients with 35µL of donors, then 5µL of the mixture were spotted onto LB plates and incubated at 37°C for 5 hours to allow mating.

5

Figure 30A shows the results from mating the donor carrying the TEM-1 beta lactamase (JA200 (F+ thr-1, leu-6, DE(trpE)5, recA, lacY, thi, gal, xyl, ara, mtl)), and designated (5) in the figure, with the recipients DH5α pNB100, designated (–), DH5α pNB102, designated (+). Here a loopful of each mating mixture was resuspended in 220µL of LB and 200µL of the cells were plated on LB Ap100Cm35 plates to select for exconjugants. As expected from Example 2, the cross between the donor of TEM-1 and the recipient with pNB100 gives efficient mating and a lawn of cells (Fig. 30A, top left, 5–), and mating the donor of TEM-1 with the recipient with pNB102 encoding the single TEM spacer shows strong inhibition of transfer of the TEM-1 gene (see Fig. 30A, 5+, top right, where only a few isolated colonies are seen). The mating of the donor carrying the TEM-1 gene with the recipient carrying pNB104 (see bottom plate, 5B, of the figure) also shows strong inhibition of transfer of the TEM-1 gene and demonstrates that in the pNB104B construct, carrying the four spacers OXA-48–SHV–TEM–CTX-M 4, the TEM spacer is active.

Figure 30B shows the results from mating the strain NCTC13440, a *K. pneumoniae* strain carrying the VIM-1, designated (2), and the strain NCTC13353, an *E. coli* strain carrying the CTX-M-15 gene, designated (4). Again a loopful of each such mating mixture was resuspended in 220µL of LB and 200µL of the cells were plated on LB Ap100Cm35 plates to select for exconjugants. The results show that these strains are able to act as donors and transfer the VIM-1 and the CTX-M-15 genes respectively to the DH5α pNB100 negative control recipient lacking Nemesis symbiotic activity (see Fig. 30B, top left and top right respectively). However transfer of the VIM-1 and the CTX-M-15 genes was found to be strongly inhibited in matings with recipients carrying spacers targeted against these genes: pNB104A and pNB104B (see plates 2A and 4B, bottom left and right respectively).

In another experiment, an equivalent mating was done to test Nemesis symbiotic activity in recipients carrying all 8 spacer sequences in the plasmid pNB108. Here cells were picked directly from the mating mixture and streaked onto LB Ap100Cm35 plates. Figure 30C plate, top left, again shows successful mating of donors 2, 4 and 5 with the recipient carrying pNB100 (see 2–, 4– and 5– in Fig. 30C). In all cases, however, mating with the recipient carrying pNB108 (see Fig. 30C, plate top right, 2/8, 4/8 and 5/8) gives strong inhibition of transfer of ampicillin resistance gene. And for comparison to a positive control

in this assay the bottom plate with 5+ (see Fig. 30C) shows strong inhibition of transfer of TEM-1 ampicillin resistance gene to the recipient carrying pNB102.

The experiments reported above provide the proof-of-concept that, in the model organism, *Escherichia coli*, DNA constructs carrying the Cas9 CRISPR region plus a spacer region
5 with sequences directed against a target region of the beta-lactamase gene can inactivate ampicillin resistance when delivered by naked DNA transformation and bacteriophage infection as well as prevent transfer of ampicillin resistance by plasmid conjugation. It is apparent that Nemesis symbiotics of the invention can be applied to pathogenic bacteria and for other antibiotic resistance genes.

10

Although the present invention has been described with reference to preferred or exemplary embodiments, those skilled in the art will recognise that various modifications and variations to the same can be accomplished without departing from the spirit and scope of the present invention and that such modifications are clearly contemplated herein. No limitation with
15 respect to the specific embodiments disclosed herein and set forth in the appended claims is intended nor should any be inferred.

All documents cited herein are incorporated by reference in their entirety.

SEQUENCE LISTING

<110> Nemesis Bioscience Ltd

<120> Therapeutic

<130> P/71337.WO01/MAR

<150> GB1406674.0

<151> 2014-04-14

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<151> 2014-08-01

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 gaagaacgtt ttccaatgat gagcactttt aaagttctgc tatgtggcgc ggtattatcc 240
 cgtgttgacg ccgggcaaga gcaactcggc cgccgcatac actattctca gaatgacttg 300

gttgagtact caccagtcac agaaaagcat cttacggatg gcatgacagt aagagaatta 360
 tgcagtgtctg ccataacat gagtgataac actgoggcca acttacttct gacaacgac 420
 ggaggaccga aggagctaac cgcttttttg cacaacatgg gggatcatgt aactgcctt 480
 gatcggtggg aaccggagct gaatgaagcc ataccaaagc acgagcgtga caccacgatg 540
 cctgcagcaa tggcaacaac gttgcgcaaa ctattaactg gcgaactact tactctagct 600
 tcccggcaac aattaataga ctggatggag gcggataaag ttgcaggacc acttctgcgc 660
 tcggcccttc cggttggtg gtttattgct gataaatctg gagccggtga gcgtgggtct 720
 cgcggtatca ttgcagcact ggggccagat ggtaagccct cccgtatcgt agttatctac 780
 acgacgggga gtcaggcaac tatggatgaa cgaaatagac agatcgctga gatagggtgcc 840
 tcactgatta agcattggta a 861

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34

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 ccttcctatt ctttctgtta aagcgttttc atgttataat aggcaaattt tagatgaaga 180
 ttattttctta ataac 195

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 aagtgccgct gtgttt 76

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 tttaagtggg atggac 76

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 20 25 30

Lys Val Leu Gly Asn Thr Asp Arg His Ser Ile Lys Lys Asn Leu Ile
 35 40 45

Gly Ala Leu Leu Phe Asp Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu
 50 55 60

Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg Lys Asn Arg Ile Cys
 65 70 75 80

Tyr Leu Gln Glu Ile Phe Ser Asn Glu Met Ala Lys Val Asp Asp Ser
 85 90 95

Phe Phe His Arg Leu Glu Glu Ser Phe Leu Val Glu Glu Asp Lys Lys
 100 105 110

His Glu Arg His Pro Ile Phe Gly Asn Ile Val Asp Glu Val Ala Tyr
 115 120 125

His Glu Lys Tyr Pro Thr Ile Tyr His Leu Arg Lys Lys Leu Val Asp
 130 135 140

Ser Thr Asp Lys Ala Asp Leu Arg Leu Ile Tyr Leu Ala Leu Ala His
 145 150 155 160

Met Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn Pro
 165 170 175

Asp Asn Ser Asp Val Asp Lys Leu Phe Ile Gln Leu Val Gln Thr Tyr
 180 185 190

Asn Gln Leu Phe Glu Glu Asn Pro Ile Asn Ala Ser Gly Val Asp Ala
 195 200 205

Lys Ala Ile Leu Ser Ala Arg Leu Ser Lys Ser Arg Arg Leu Glu Asn
 210 215 220

Leu Ile Ala Gln Leu Pro Gly Glu Lys Lys Asn Gly Leu Phe Gly Asn
 225 230 235 240

Leu Ile Ala Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe
 245 250 255

Asp Leu Ala Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp
 260 265 270

Asp Asp Leu Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp
 275 280 285

Leu Phe Leu Ala Ala Lys Asn Leu Ser Asp Ala Ile Leu Leu Ser Asp
 290 295 300

Ile Leu Arg Val Asn Thr Glu Ile Thr Lys Ala Pro Leu Ser Ala Ser
 305 310 315 320

Met Ile Lys Arg Tyr Asp Glu His His Gln Asp Leu Thr Leu Leu Lys
 325 330 335

Ala Leu Val Arg Gln Gln Leu Pro Glu Lys Tyr Lys Glu Ile Phe Phe
 340 345 350

Asp Gln Ser Lys Asn Gly Tyr Ala Gly Tyr Ile Asp Gly Gly Ala Ser
 355 360 365

Gln Glu Glu Phe Tyr Lys Phe Ile Lys Pro Ile Leu Glu Lys Met Asp
 370 375 380

Gly Thr Glu Glu Leu Leu Val Lys Leu Asn Arg Glu Asp Leu Leu Arg
 385 390 395 400

Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro His Gln Ile His Leu
 405 410 415

Gly Glu Leu His Ala Ile Leu Arg Arg Gln Glu Asp Phe Tyr Pro Phe
 420 425 430

Leu Lys Asp Asn Arg Glu Lys Ile Glu Lys Ile Leu Thr Phe Arg Ile
 435 440 445

Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn Ser Arg Phe Ala Trp
 450 455 460

Met Thr Arg Lys Ser Glu Glu Thr Ile Thr Pro Trp Asn Phe Glu Glu
 465 470 475 480

Val Val Asp Lys Gly Ala Ser Ala Gln Ser Phe Ile Glu Arg Met Thr
 485 490 495

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Asn Phe Asp Lys Asn Leu Pro Asn Glu Lys Val Leu Pro Lys His Ser
    500                                505                                510

Leu Leu Tyr Glu Tyr Phe Thr Val Tyr Asn Glu Leu Thr Lys Val Lys
    515                                520                                525

Tyr Val Thr Glu Gly Met Arg Lys Pro Ala Phe Leu Ser Gly Glu Gln
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Lys Lys Ala Ile Val Asp Leu Leu Phe Lys Thr Asn Arg Lys Val Thr
    545                                550                                555                                560

Val Lys Gln Leu Lys Glu Asp Tyr Phe Lys Lys Ile Glu Cys Phe Asp
    565                                570                                575

Ser Val Glu Ile Ser Gly Val Glu Asp Arg Phe Asn Ala Ser Leu Gly
    580                                585                                590

Thr Tyr His Asp Leu Leu Lys Ile Ile Lys Asp Lys Asp Phe Leu Asp
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Asn Glu Glu Asn Glu Asp Ile Leu Glu Asp Ile Val Leu Thr Leu Thr
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Leu Phe Glu Asp Arg Glu Met Ile Glu Glu Arg Leu Lys Thr Tyr Ala
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His Leu Phe Asp Asp Lys Val Met Lys Gln Leu Lys Arg Arg Arg Tyr
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Thr Gly Trp Gly Arg Leu Ser Arg Lys Leu Ile Asn Gly Ile Arg Asp
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Lys Gln Ser Gly Lys Thr Ile Leu Asp Phe Leu Lys Ser Asp Gly Phe
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Ala Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp Ser Leu Thr Phe
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Lys Glu Asp Ile Gln Lys Ala Gln Val Ser Gly Gln Gly Asp Ser Leu
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Ile Leu Gln Thr Val Lys Val Val Asp Glu Leu Val Lys Val Met Gly
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Arg His Lys Pro Glu Asn Ile Val Ile Glu Met Ala Arg Glu Asn Gln
 755 760 765

Thr Thr Gln Lys Gly Gln Lys Asn Ser Arg Glu Arg Met Lys Arg Ile
 770 775 780

Glu Glu Gly Ile Lys Glu Leu Gly Ser Gln Ile Leu Lys Glu His Pro
 785 790 795 800

Val Glu Asn Thr Gln Leu Gln Asn Glu Lys Leu Tyr Leu Tyr Tyr Leu
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Gln Asn Gly Arg Asp Met Tyr Val Asp Gln Glu Leu Asp Ile Asn Arg
 820 825 830

Leu Ser Asp Tyr Asp Val Asp His Ile Val Pro Gln Ser Phe Leu Lys
 835 840 845

Asp Asp Ser Ile Asp Asn Lys Val Leu Thr Arg Ser Asp Lys Asn Arg
 850 855 860

Gly Lys Ser Asp Asn Val Pro Ser Glu Glu Val Val Lys Lys Met Lys
 865 870 875 880

Asn Tyr Trp Arg Gln Leu Leu Asn Ala Lys Leu Ile Thr Gln Arg Lys
 885 890 895

Phe Asp Asn Leu Thr Lys Ala Glu Arg Gly Gly Leu Ser Glu Leu Asp
 900 905 910

Lys Ala Gly Phe Ile Lys Arg Gln Leu Val Glu Thr Arg Gln Ile Thr
 915 920 925

Lys His Val Ala Gln Ile Leu Asp Ser Arg Met Asn Thr Lys Tyr Asp
 930 935 940

Glu Asn Asp Lys Leu Ile Arg Glu Val Lys Val Ile Thr Leu Lys Ser
 945 950 955 960

Lys Leu Val Ser Asp Phe Arg Lys Asp Phe Gln Phe Tyr Lys Val Arg
 965 970 975

Glu Ile Asn Asn Tyr His His Ala His Asp Ala Tyr Leu Asn Ala Val
 980 985 990

Val Gly Thr Ala Leu Ile Lys Lys Tyr Pro Lys Leu Glu Ser Glu Phe
 995 1000 1005

Val Tyr Gly Asp Tyr Lys Val Tyr Asp Val Arg Lys Met Ile Ala
 1010 1015 1020

Lys Ser Glu Gln Glu Ile Gly Lys Ala Thr Ala Lys Tyr Phe Phe
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Tyr Ser Asn Ile Met Asn Phe Phe Lys Thr Glu Ile Thr Leu Ala
 1040 1045 1050

Asn Gly Glu Ile Arg Lys Arg Pro Leu Ile Glu Thr Asn Gly Glu
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Thr Gly Glu Ile Val Trp Asp Lys Gly Arg Asp Phe Ala Thr Val
 1070 1075 1080

Arg Lys Val Leu Ser Met Pro Gln Val Asn Ile Val Lys Lys Thr
 1085 1090 1095

Glu Val Gln Thr Gly Gly Phe Ser Lys Glu Ser Ile Leu Pro Lys
 1100 1105 1110

Arg Asn Ser Asp Lys Leu Ile Ala Arg Lys Lys Asp Trp Asp Pro
 1115 1120 1125

Lys Lys Tyr Gly Gly Phe Asp Ser Pro Thr Val Ala Tyr Ser Val
 1130 1135 1140

Leu Val Val Ala Lys Val Glu Lys Gly Lys Ser Lys Lys Leu Lys
 1145 1150 1155

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Phe Glu	Lys Asn Pro Ile Asp	Phe Leu Glu Ala Lys	Gly Tyr Lys
1175	1180	1185	
Glu Val	Lys Lys Asp Leu Ile	Ile Lys Leu Pro Lys	Tyr Ser Leu
1190	1195	1200	
Phe Glu	Leu Glu Asn Gly Arg	Lys Arg Met Leu Ala	Ser Ala Gly
1205	1210	1215	
Glu Leu	Gln Lys Gly Asn Glu	Leu Ala Leu Pro Ser	Lys Tyr Val
1220	1225	1230	
Asn Phe	Leu Tyr Leu Ala Ser	His Tyr Glu Lys Leu	Lys Gly Ser
1235	1240	1245	
Pro Glu	Asp Asn Glu Gln Lys	Gln Leu Phe Val Glu	Gln His Lys
1250	1255	1260	
His Tyr	Leu Asp Glu Ile Ile	Glu Gln Ile Ser Glu	Phe Ser Lys
1265	1270	1275	
Arg Val	Ile Leu Ala Asp Ala	Asn Leu Asp Lys Val	Leu Ser Ala
1280	1285	1290	
Tyr Asn	Lys His Arg Asp Lys	Pro Ile Arg Glu Gln	Ala Glu Asn
1295	1300	1305	
Ile Ile	His Leu Phe Thr Leu	Thr Asn Leu Gly Ala	Pro Ala Ala
1310	1315	1320	
Phe Lys	Tyr Phe Asp Thr Thr	Ile Asp Arg Lys Arg	Tyr Thr Ser
1325	1330	1335	
Thr Lys	Glu Val Leu Asp Ala	Thr Leu Ile His Gln	Ser Ile Thr
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20

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cccggcaaca attaatagac

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<211> 81

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<223> Adaptor sequence for dual targets

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<223> Cleaved tracrRNA after first processing event

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<223> cleaved pre-crRNA

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 <223> crRNA complementary sequence within target beta-lactamase DNA sequence

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<210> 137
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 <212> DNA
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 <223> CR90-containing target beta-lactamase DNA sequence anti-protospacer strand

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<210> 138
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 <212> DNA
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 <223> CR90-containing target beta-lactamase DNA sequence protospacer strand

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<220>
 <223> crRNA complementary sequence within target beta-lactamase DNA sequence - reverse strand

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 <212> DNA
 <213> Streptococcus pyogenes

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<210> 142
 <211> 10
 <212> DNA
 <213> *Streptococcus pyogenes*

<400> 142
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<210> 143
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 <212> DNA
 <213> *Streptococcus pyogenes*

<400> 143
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<210> 144
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 <212> DNA
 <213> Artificial

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 <223> Fragment 3, amplified from genomic DNA of *S. pyogenes* strain SF370

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<211> 9578

<212> DNA

<213> Artificial

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<223> Sequence of the final construct pNB100

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<210> 146

<211> 386

<212> DNA

<213> Artificial

<220>

<223> Amplified sequence with primer NB018 and NB019 from pNB100 as template

<400> 146

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atggtcccaa aacttgccga tgataacttg agaaagaggg ttaataccag cagtcggata      120
ccttcctatt ctttctgtta aagcgttttc atgttataat aggcaaattt tagatgaaga      180
ttattttctta ataactaaaa atatggtata atactcttaa taaatgcagt aatacagggg      240
cttttcaaga ctgaagtcta gctgagacaa atagtgcgat tacgaaattt ttagacaaa      300
aatagtctac gaggttttag agctatgctg ttttgaatgg tcccaaaact gaagagcgtc      360
tcggacgcag cgctcttcgt tttaga                                           386

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<210> 147

<211> 744

<212> DNA

<213> Artificial

<220>

<223> Modified CRISPR array in pNB200

<400> 147

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tcaagactga agtctagctg agacaaatag tgcgattacg aaatttttta gacaaaaata      180
gtctacgagg ttttagagct atgctatttt gaatgggtccc aaaactgaga cctgctgcgg      240
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tagagctatg ctgttttgaa tgggtccaaa acaacattgc cgatgataac ttgagaaaga      660
gggttaatac cagcagtcgg ataccttcct attctttctg ttaaagcgtt ttcattgtat      720

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aataggcaaa agaagagtag tgtg

744

<210> 148

<211> 23

<212> DNA

<213> Artificial

<220>

<223> Primer sequence

<400> 148

ccaactacct ccccttgctt aac

23

<210> 149

<211> 18

<212> DNA

<213> Artificial

<220>

<223> Primer sequence

<400> 149

ggtgactgat ggccacgt

18

<210> 150

<211> 23

<212> DNA

<213> Artificial

<220>

<223> Primer sequence

<400> 150

gggctggcaa gccacgtttg gtg

23

<210> 151

<211> 23

<212> DNA

<213> Artificial

<220>

<223> Primer sequence

<400> 151

ccgggagctg catgtgtcag agg

23

<210> 152

<211> 43

<212> DNA

<213> Artificial

<220>

<223> Primer sequence

<400> 152

attgaaaaag gaagagtatg gaattgccca atattatgca ccc

43

<210> 153

<211> 38

<212> DNA

<213> Artificial

<220>

<223> Primer sequence

<400> 153

agtcccgcta ggtctcaacc gtcagcgag cttgtcgg

38

<210> 154

<211> 42

<212> DNA

<213> Artificial

<220>

<223> Primer sequence

<400> 154

attgaaaaag gaagagtatg cgtgtattag cttatcggc tg

42

<210> 155

<211> 55

<212> DNA

<213> Artificial

<220>

<223> Primer sequence

<400> 155

agtcccgcta ggtctcaacc gctaggggaat aattttttcc tgtttgagca cttct

55

<210> 156

<211> 47

<212> DNA

<213> Artificial

<220>

<223> Primer sequence

<400> 156

attgaaaaag gaagagtatg cgttattttc gcctgtgtat tatctcc

47

<210> 157
<211> 41
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 157
agtcccgcta ggtctcaacc gtttagcgttg ccagtgctcg a 41

<210> 158
<211> 57
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 158
attgaaaaag gaagagtatg ttaaaagtta ttagtagttt attggtctac atgaccg 57

<210> 159
<211> 48
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 159
agtcccgcta ggatgacctg gctgaccgct actcggcgac tgagcgat 48

<210> 160
<211> 43
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 160
attgaaaaag gaagagtatg tcaactgtatc gccgtctagt tct 43

<210> 161
<211> 39
<212> DNA
<213> Artificial

<220>
<223> KPC-3 reverse primer

<400> 161
agtccecgcta ggtctcaacc gttactgccc gttgacgcc 39

<210> 162
<211> 60
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 162
attgaaaaag gaagagtatg agcaagttat ctgtattctt tatatctttg ttttgttagca 60

<210> 163
<211> 61
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 163
agtccecgcta ggtctcaacc gttagtgtct tagttttgat ggttttttac tttcgtttaa 60

c 61

<210> 164
<211> 44
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 164
attgaaaaag gaagagtatg gttaaaaaat cactgcgcga gttc 44

<210> 165
<211> 47
<212> DNA
<213> Artificial

<220>
<223> Primer sequence

<400> 165
agtccecgcta ggtctcaacc gttacaaacc gtcggtgacg atttttag 47

<210> 166
<211> 834

<212> DNA

<213> Unknown

<220>

<223> NDM-1 sequence

<400> 166

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ggcggatttc accgggcatg caccgctca gcatcaatgc agcggctaata gcggtgctca      780
gcttcgcgac cgggtgcata atattgggca attccatact ctcccttttt caat          834

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<210> 167

<211> 819

<212> DNA

<213> Unknown

<220>

<223> OXA-48 sequence

<400> 167

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gttcaacca accgaccac cagccaatct taggttcgat tctagtcgag tatccagttt      180
tagccogaat aatatagtca ccattggctt cggtcagcat ggcttgtttg acaatacgct      240
ggctgcgctc cgatacgtgt aacttattgt gatacagctt tcttaaaaag ctgatttgct      300
ccgtggccga aattogaata ccaccgtcga gccagaaact gtctacattg cccgaaatgt      360

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cctcattacc ataatcgaaa gcatgtagca tcttgetcat acgtgcctcg ccaatttggc 420
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 tttcttgcca ttcttttgc accgcaggca ttccgataat cgatgccacc aaaaacacag 780
 ccgataaggc taatacacgc atactcttcc tttttcaat 819

<210> 168

<211> 882

<212> DNA

<213> Unknown

<220>

<223> SHV-1 sequence

<400> 168

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 tttgctcaag cggtcgggg ctggcgtgta ccgccagcgg cagggtggct aacagggaga 840
 taatacacag gcgaaaataa cgcatactct tcctttttca at 882

<210> 169
 <211> 822
 <212> DNA
 <213> Unknown

<220>
 <223> VIM-1 sequence

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 gctccccgga atgggctaac ggacttgcca cagccatgac agacgcggtc atgtagacca 780
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<210> 170
 <211> 903
 <212> DNA
 <213> Unknown

<220>
 <223> KPC-3 sequence

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Claims

1. A delivery vehicle for introducing a polynucleotide into an antibiotic-resistant microorganism, in which the delivery vehicle comprises a recombinant polynucleotide for inactivation of DNA carrying a gene encoding an antibiotic resistance enzyme which confers antibiotic resistance to the antibiotic-resistant microorganism, and in which the recombinant polynucleotide comprises a clustered regularly interspaced short palindromic repeat (CRISPR) array nucleic acid sequence having or transcribing an RNA guide molecule with a spacer sequence sufficiently complementary to a target DNA sequence of the antibiotic resistance gene for the antibiotic resistance gene to be targeted and inactivated in the presence of a CRISPR associated (Cas) DNA-binding polypeptide or a functional equivalent or a modified version thereof, thereby sensitising the microorganism to the antibiotic, wherein the delivery vehicle is a non-virulent or a lysogenic bacteriophage.
2. The delivery vehicle according to claim 1, in which the RNA guide molecule mediates the binding of the Cas DNA-binding polypeptide or its functional equivalent or its modified version to the antibiotic resistance gene.
3. The delivery vehicle according to either of claim 1 or claim 2, further comprising a nucleic acid sequence which encodes the Cas DNA-binding polypeptide or its functional equivalent or its modified version.
4. The delivery vehicle according to any of the preceding claims, in which the Cas DNA-binding polypeptide is Cas9 or a functional equivalent or a modified version thereof.
5. The delivery vehicle according to any of the preceding claims, comprising a further RNA guide molecule which targets a gene involved in pathogenicity or other aspects of microbial metabolism, for example a gene involved in bacterial metabolism for biofilm production.
6. The delivery vehicle according to any of the preceding claims, in which the Cas DNA-binding polypeptide is modified to comprise a recombinase catalytic domain such that the modified Cas DNA-binding polypeptide inactivates the target DNA sequence by creating a deletion and sealing the target sequence but does not generate a double-stranded break in the target DNA sequence.

7. The delivery vehicle according to any of the preceding claims, in which the CRISPR array nucleic acid sequence has or transcribes additional RNA guide molecules each comprising a spacer sequence sufficiently complementary to a target sequence of the antibiotic resistance gene or one or more additional antibiotic resistance genes.
8. The delivery vehicle according to any of the preceding claims, in which the CRISPR array nucleic acid sequence has or transcribes one or more RNA guide molecules each comprising a spacer sequence sufficiently complementary to a target sequence of one or more beta-lactamase genes.
9. The delivery vehicle according to claim 8, in which the one or more RNA guide molecules target one or more or all of the genes selected from the group consisting of : NDM, VIM, IMP, KPC, OXA, TEM, SHV, CTX, OKP, LEN, GES, MIR, ACT, ACC, CMY, LAT, and FOX.
10. The delivery vehicle according to any of the preceding claims, in which the or each RNA guide molecule is transcribed from its own promoter sequence.
11. The delivery vehicle according to any of the preceding claims, in which the antibiotic resistance gene is located on a chromosome, or on an extrachromosomal replicating DNA molecule (a replicon) including a plasmid or a bacteriophage.
12. The delivery vehicle according to any of the preceding claims, further comprising a nucleotide sequence which encodes a gene conferring a selective advantage to the microorganism.
13. A composition comprising the delivery vehicle according to any of claims 1 to 12.
14. The composition according to claim 13, in which the composition is a pharmaceutical composition, a non-pathogenic microorganism such as a commensal bacterium for example in a probiotic formulation, or a dietary supplement.
15. The composition according to either of claims 13 or 15, formulated for topical, enteral or parenteral administration.
16. The composition according to any of claims 13 to 15, for use as a medicament.

17. The composition according to any of claims 13 to 15, for use in the treatment or prevention of an infection caused by an antibiotic-resistant microorganism comprising an antibiotic resistance gene targeted by the RNA guide molecule of the recombinant polynucleotide.

18. A method of treating or preventing an infection in a subject caused by an antibiotic-resistant microorganism comprising an antibiotic resistance gene, in which the method comprises the step of introducing into the microorganism a therapeutically effective amount of the composition according to any of claims 13 to 15 where the RNA guide molecule targets the antibiotic resistance gene, thereby inactivating the antibiotic resistance gene and sensitising the microorganism to the antibiotic.

19. The method according to claim 18, in which the composition is administered topically or orally.

20. The method according to either of claims 18 or 19, in which the subject is a fish, a bird, a reptile or a mammal (such as a human).

21. The method according to any of claims 17 to 19, in which the delivery vehicle is transferred from the antibiotic-resistant microorganism directly into another microorganism (such as antibiotic-resistant microorganism) by plasmid conjugation or bacteriophage infection.

22. The method according to any of claims 17 to 21, further comprising a step of simultaneously or subsequently administering to the subject an antibiotic to which microorganism has become sensitised.

23. A method of inactivating antibiotic resistance in an antibiotic-resistant microorganism, the method comprising introducing into the microorganism the delivery vehicle according to any of claims 1 to 12.

24. A host cell comprising the recombinant polynucleotide as defined in any of claims 1 to 12.

25. The host cell according to claim 24, in which the host cell is a commensal bacterium.

26. A delivery vehicle, composition, composition for use, methods, or host cell as described herein with reference to the accompanying figures.

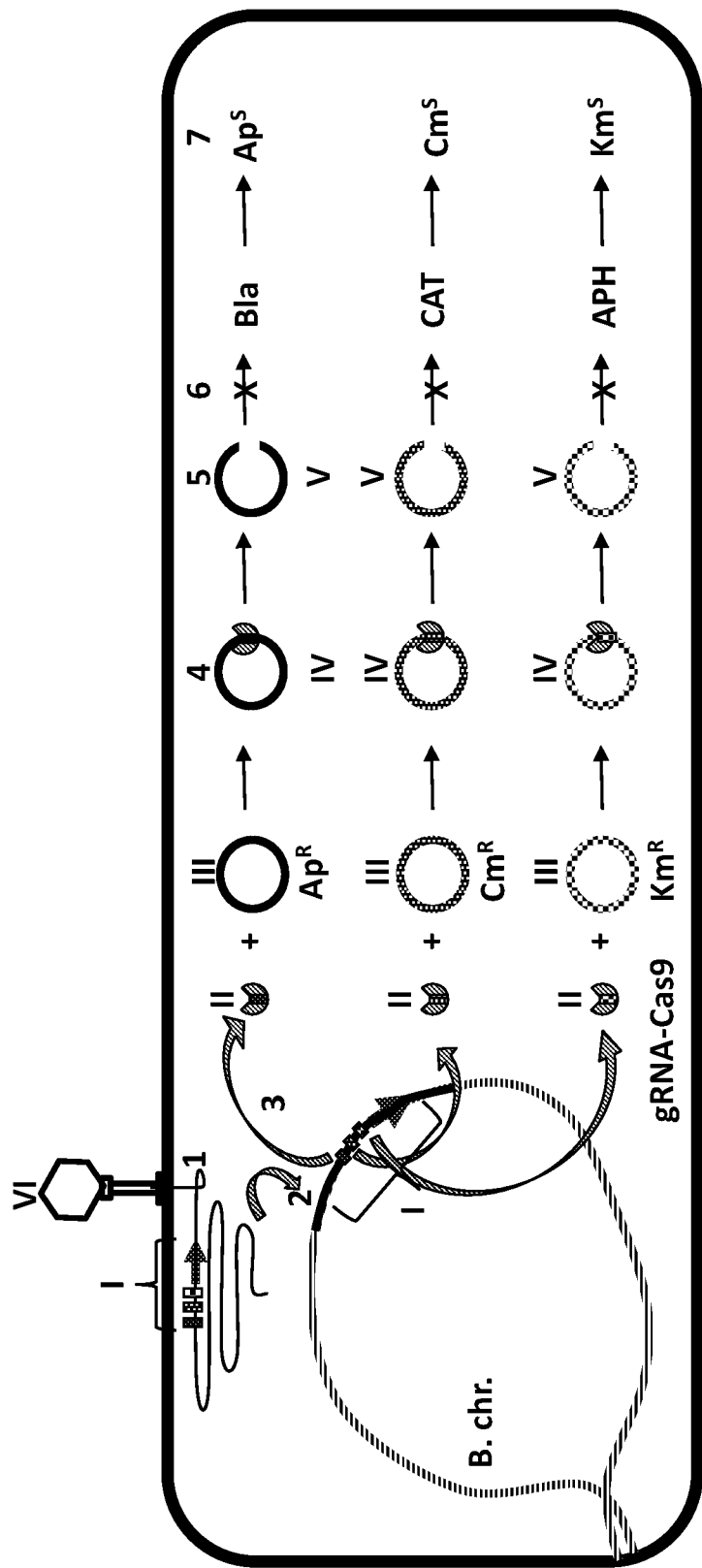


Fig. 1

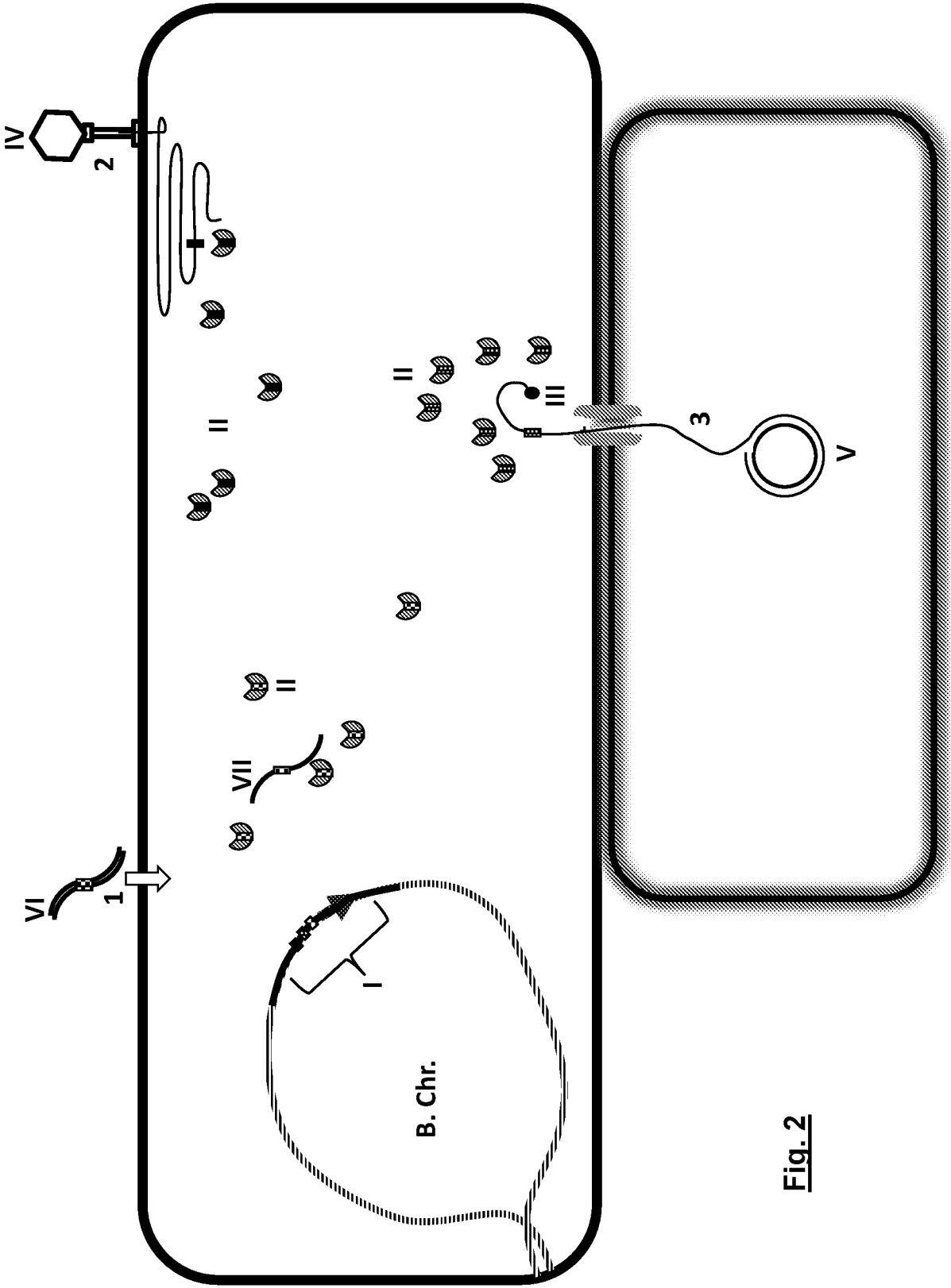


Fig. 2

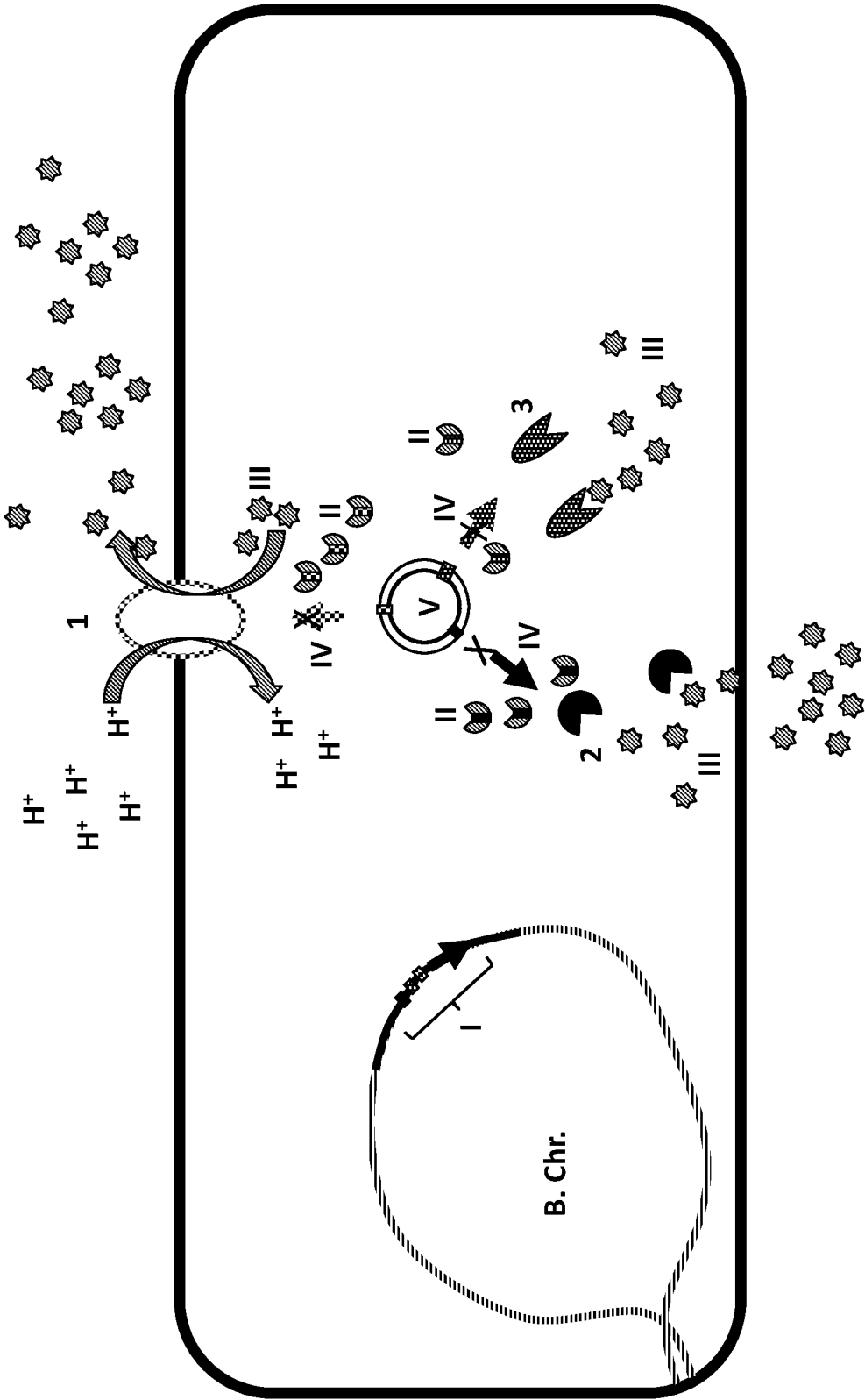


Fig. 3

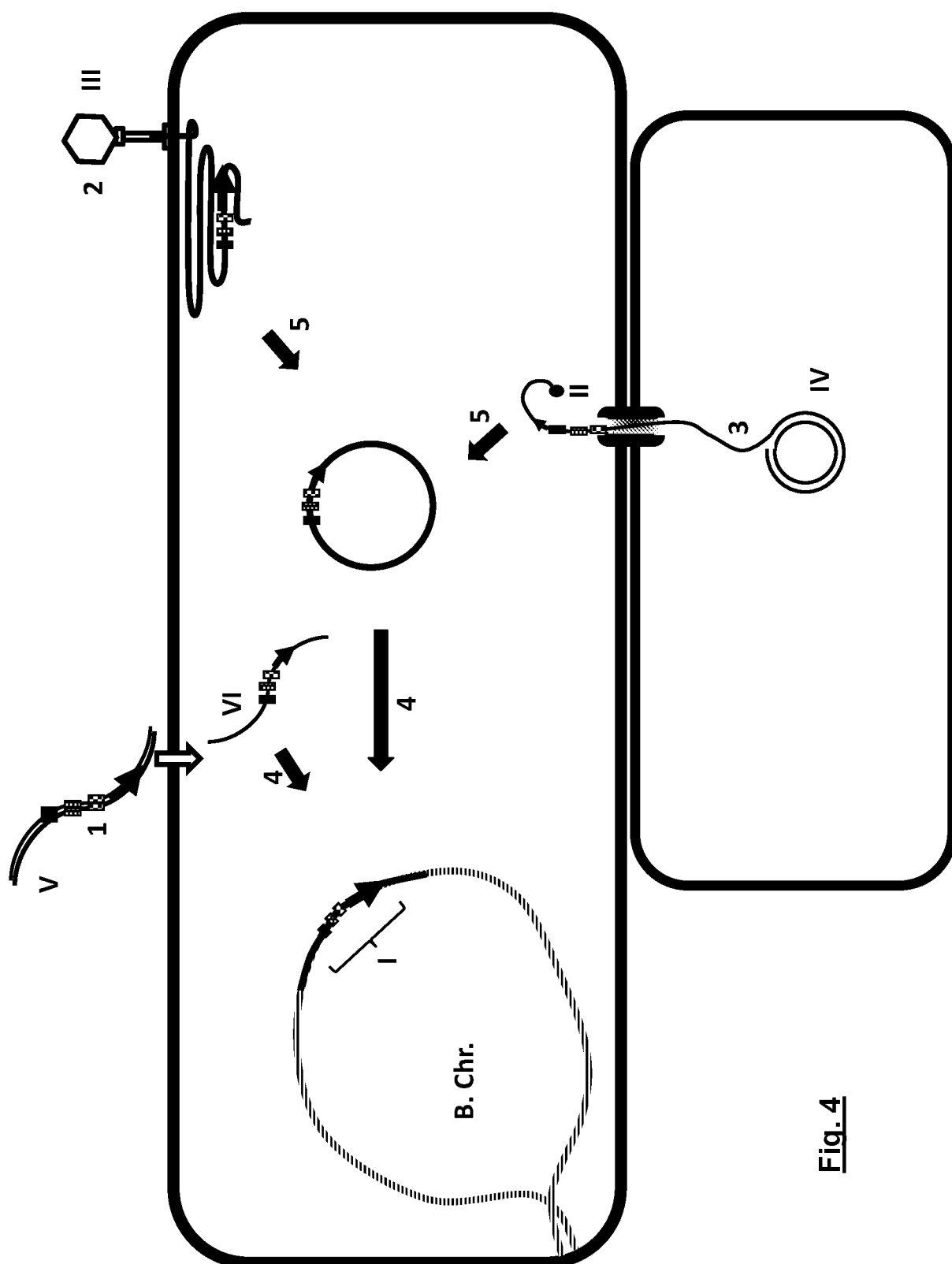


Fig. 4

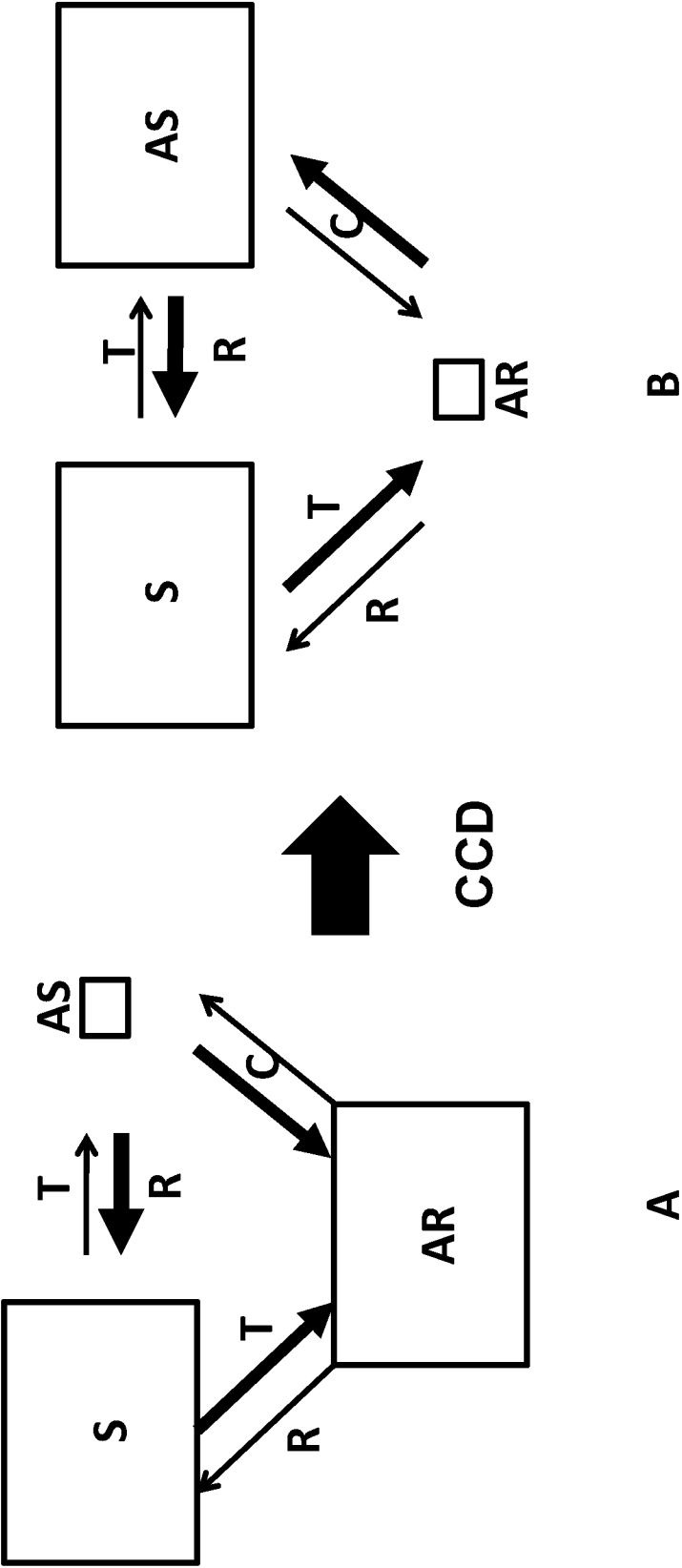


Fig. 5

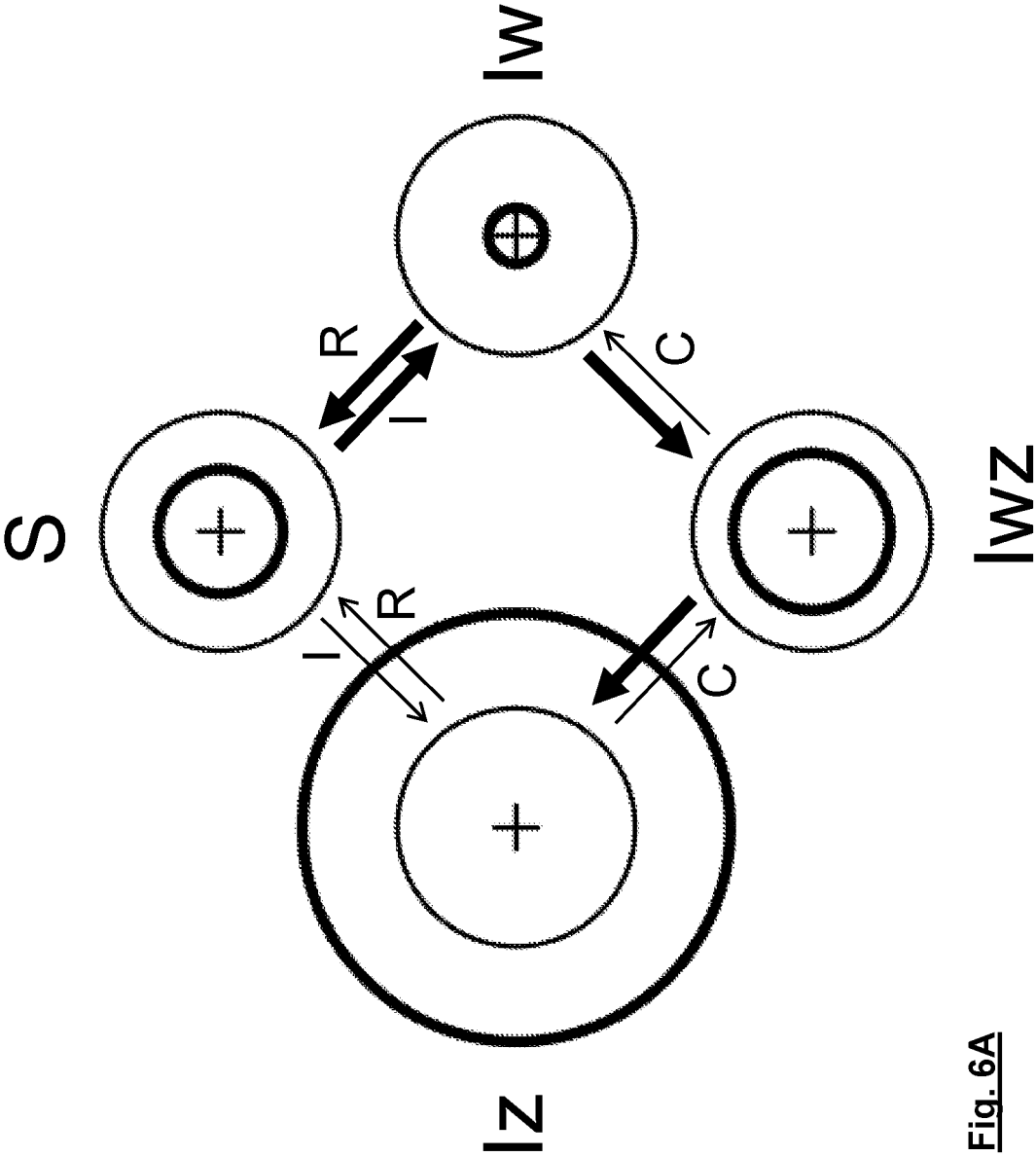


Fig. 6A

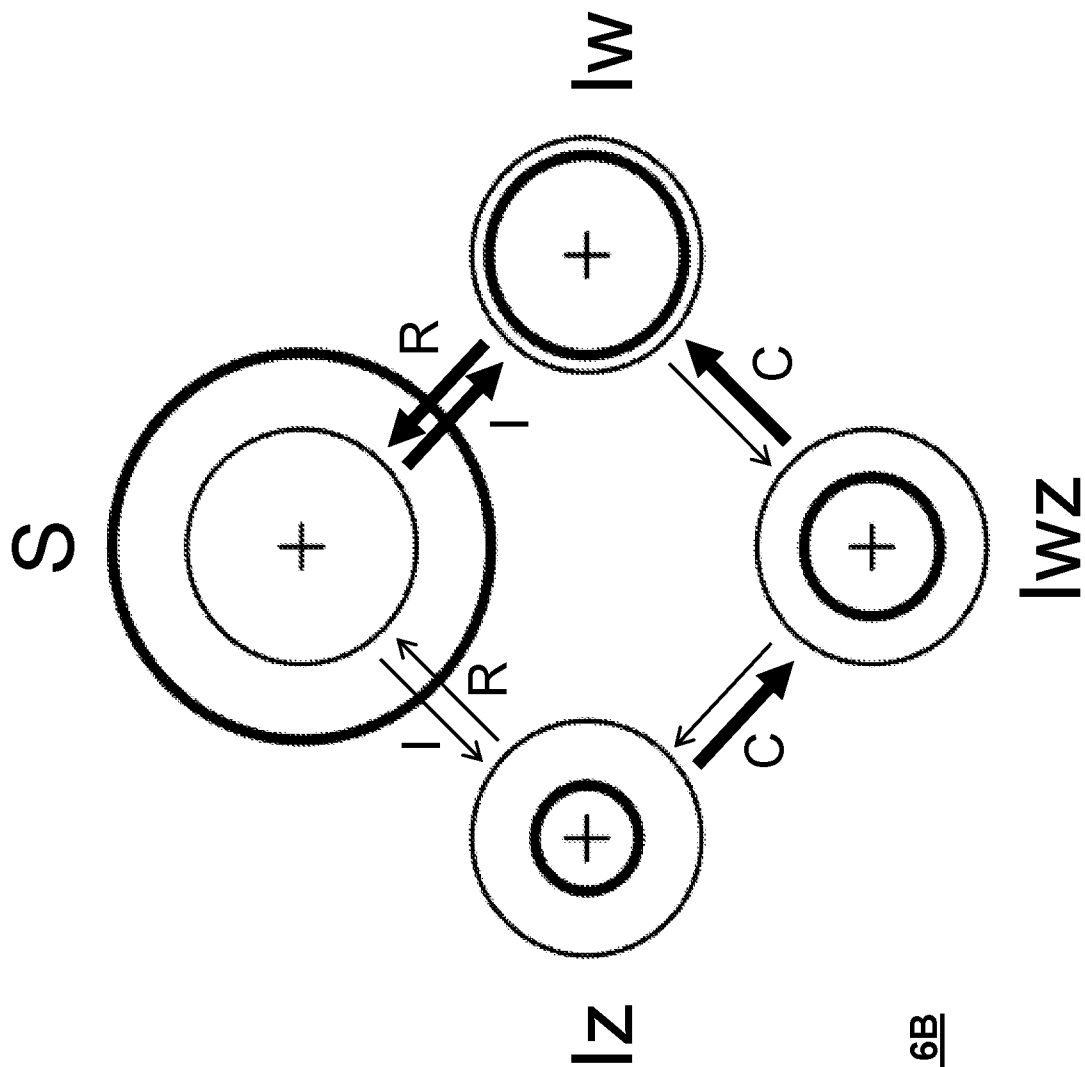


Fig. 6B

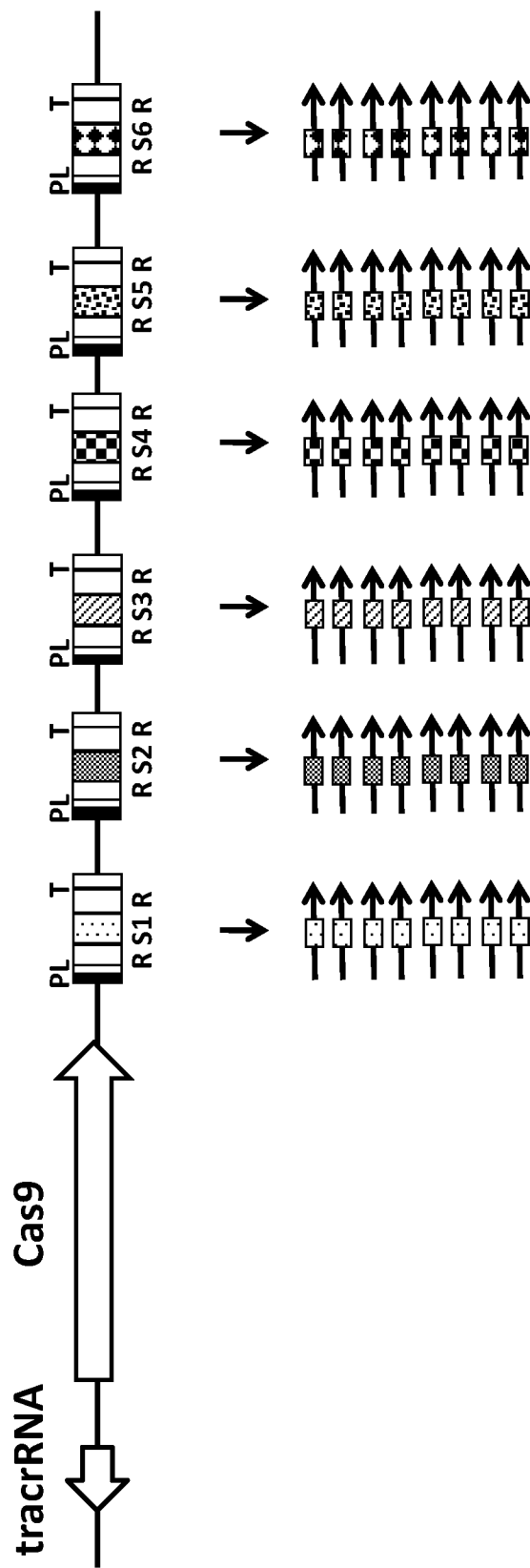


Fig. 7

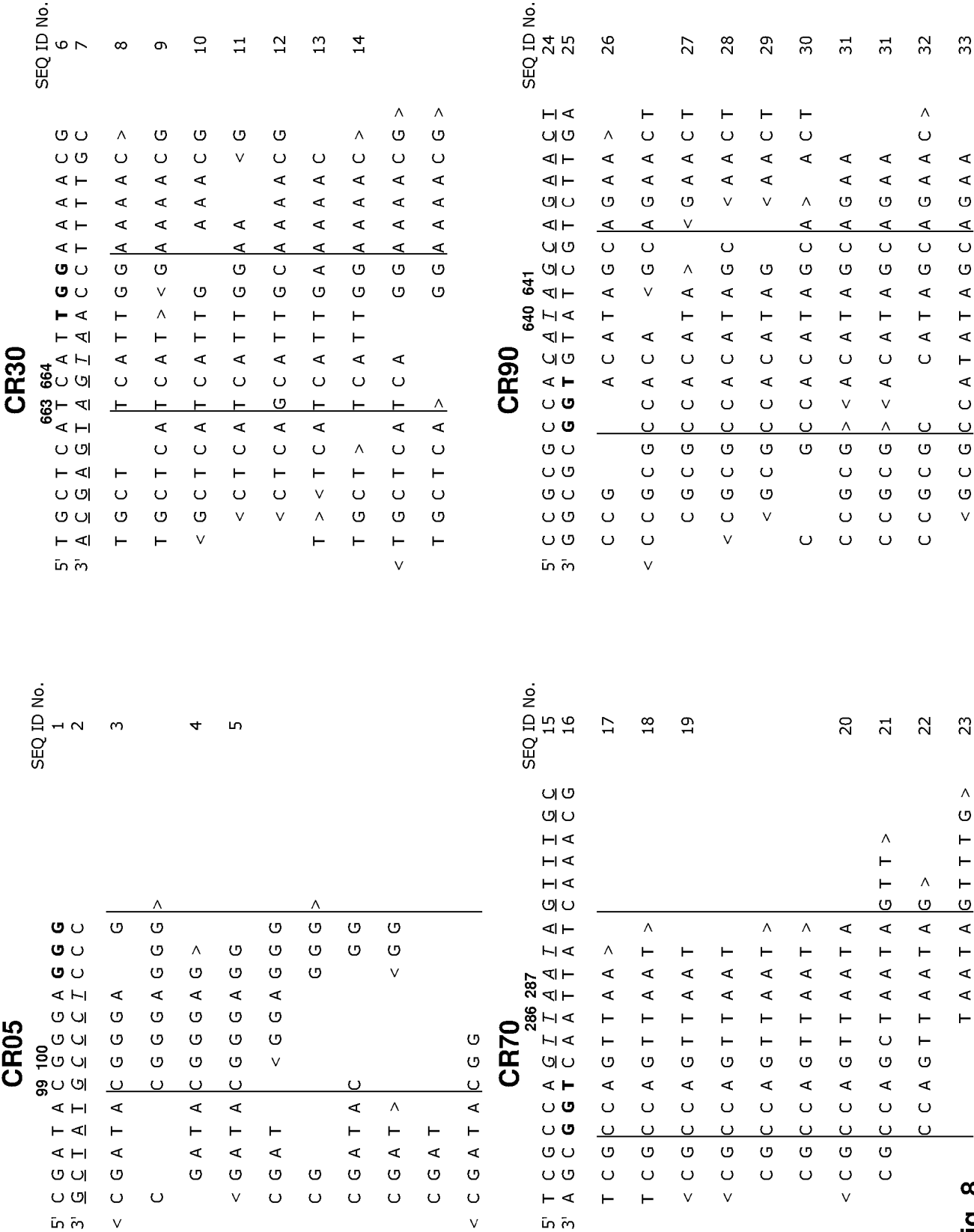


Fig. 8

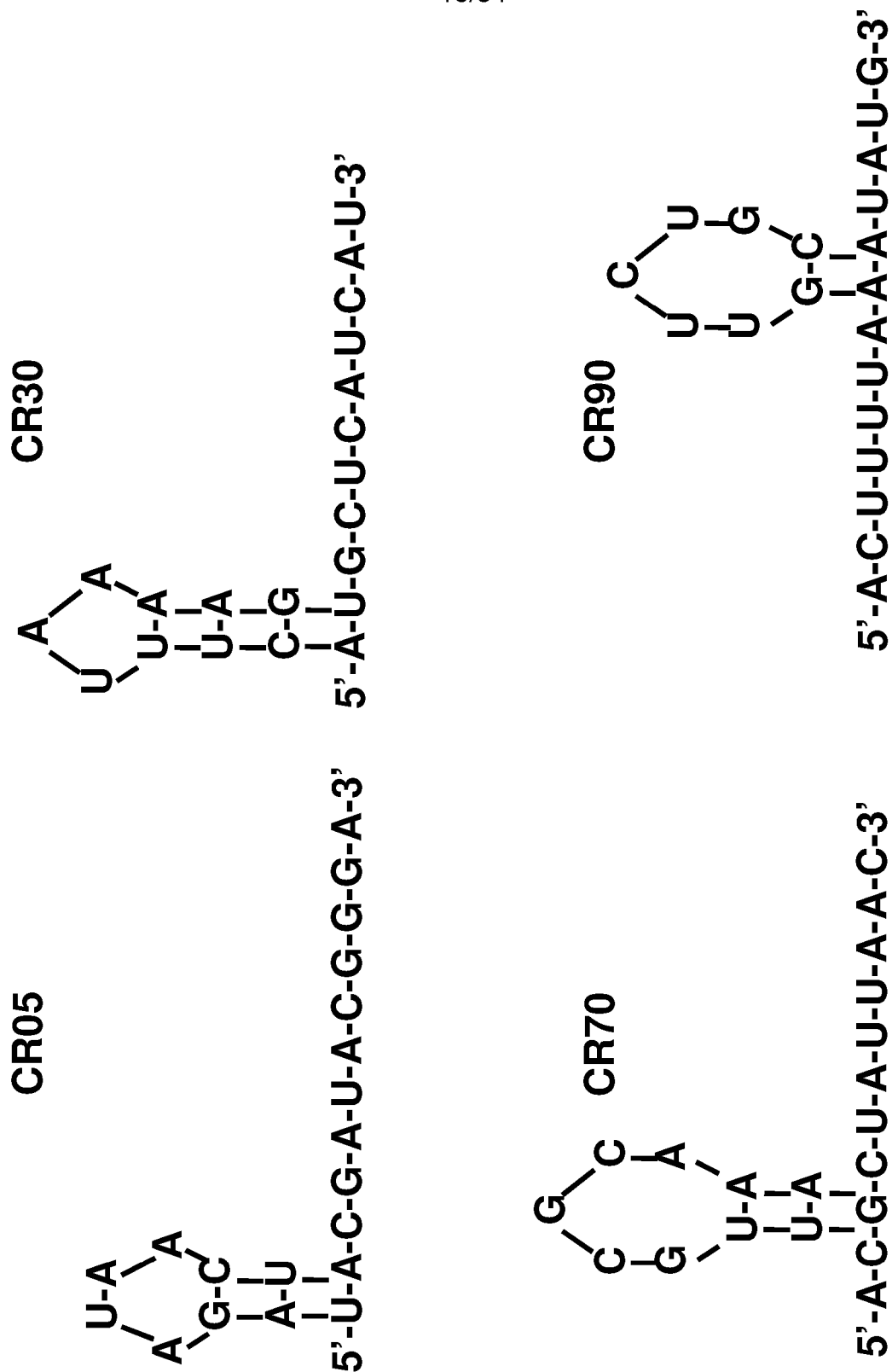


Fig. 9

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Fig. 10

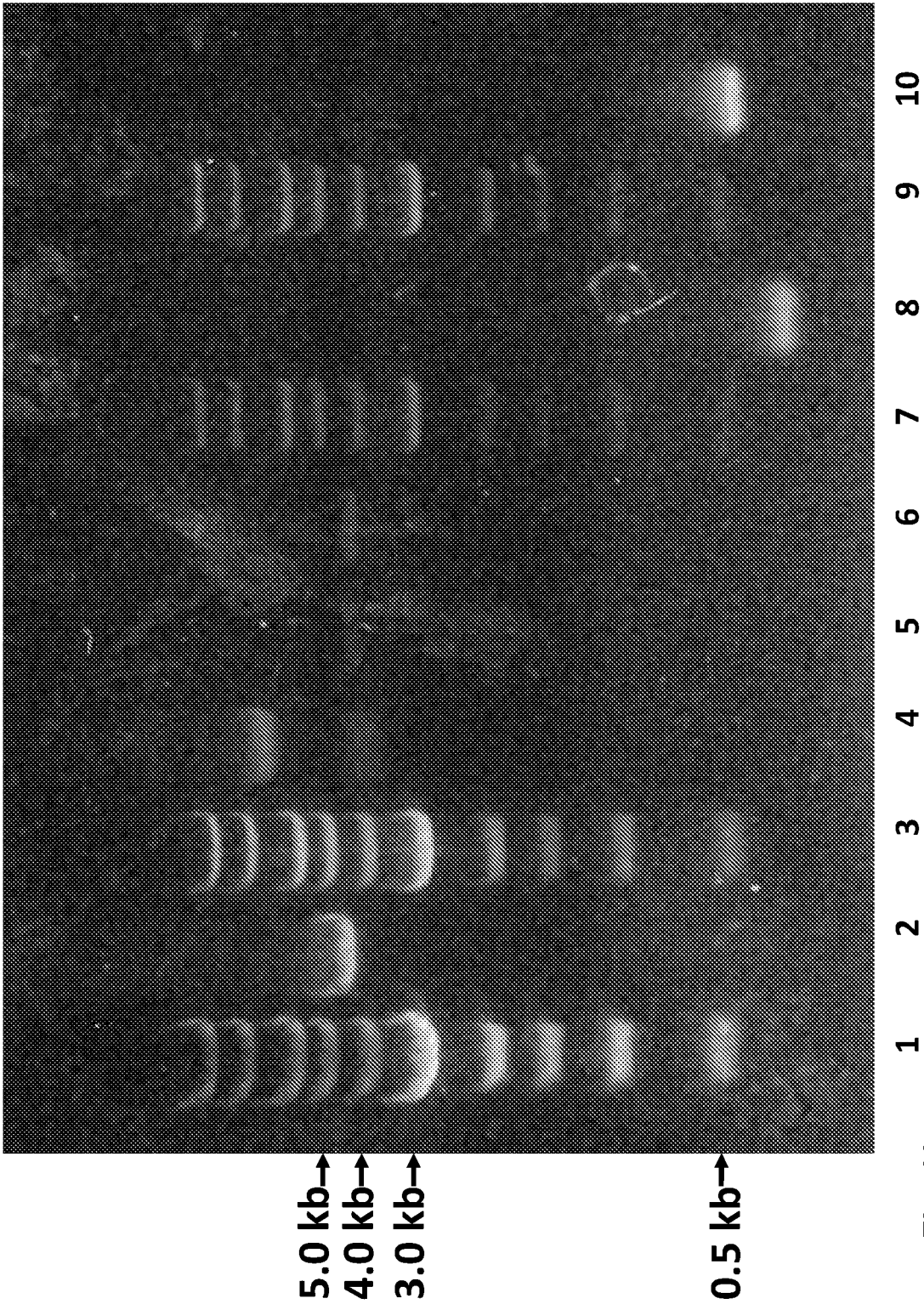


Fig. 11

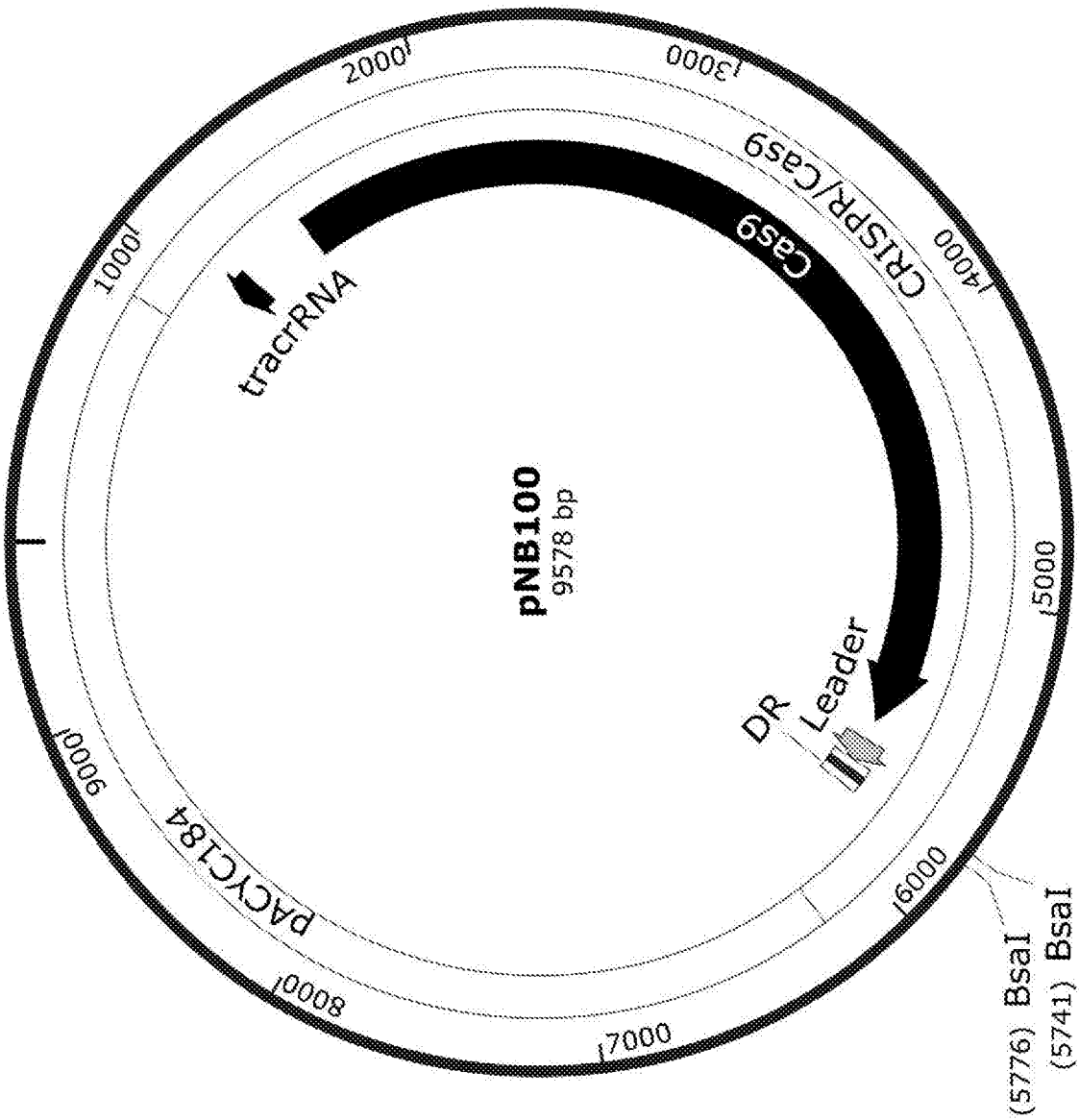


Fig. 12

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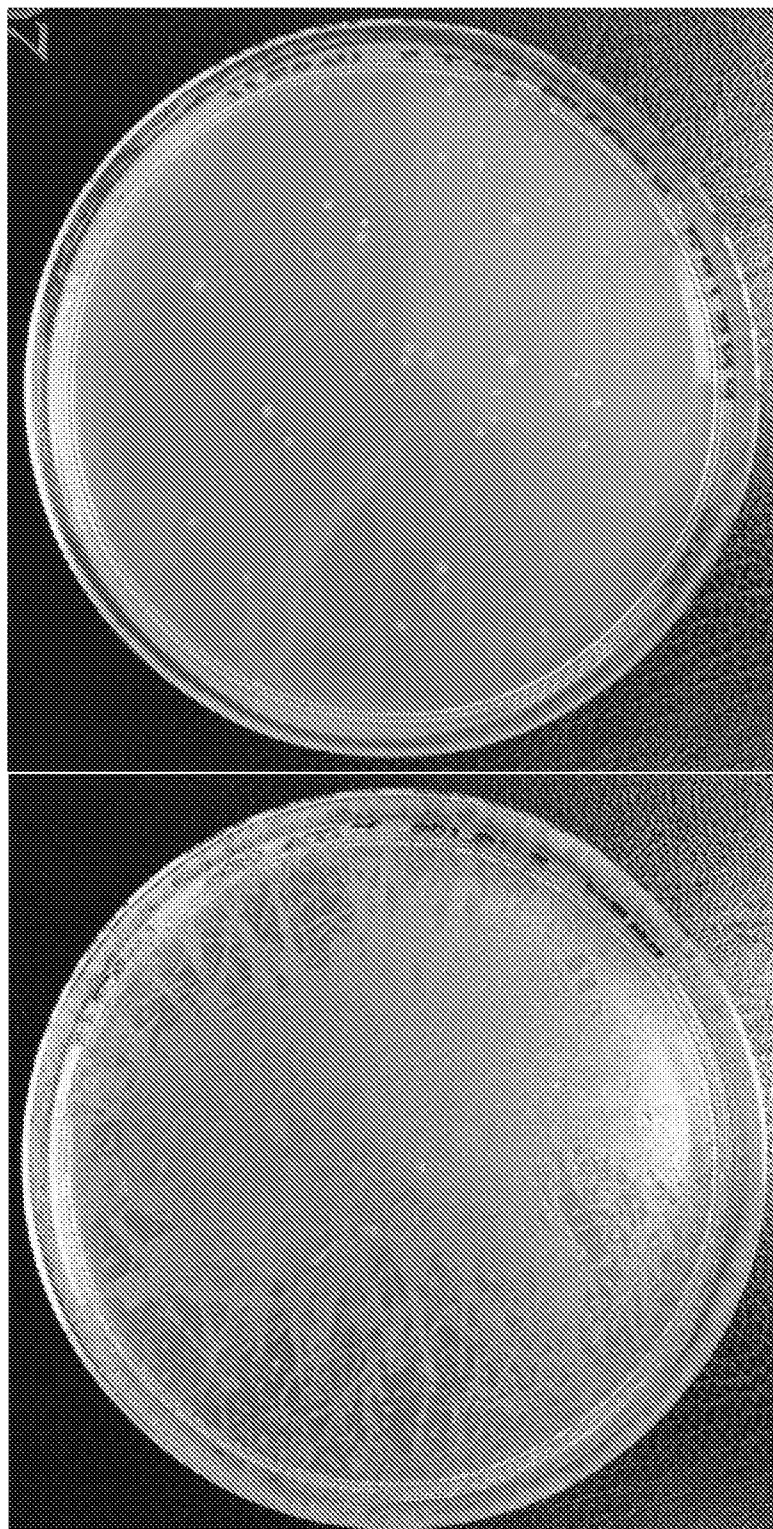


Fig. 13

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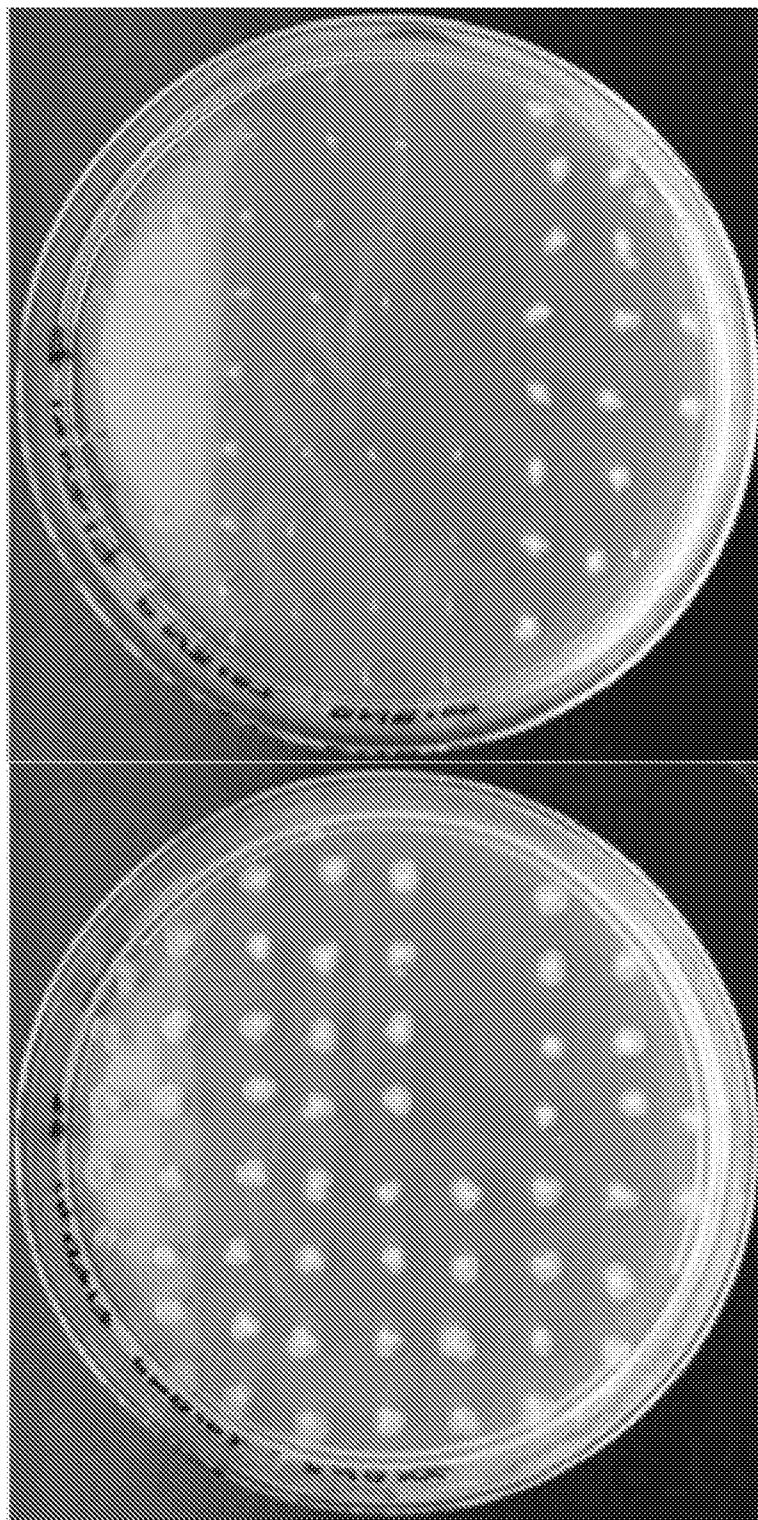


Fig. 14

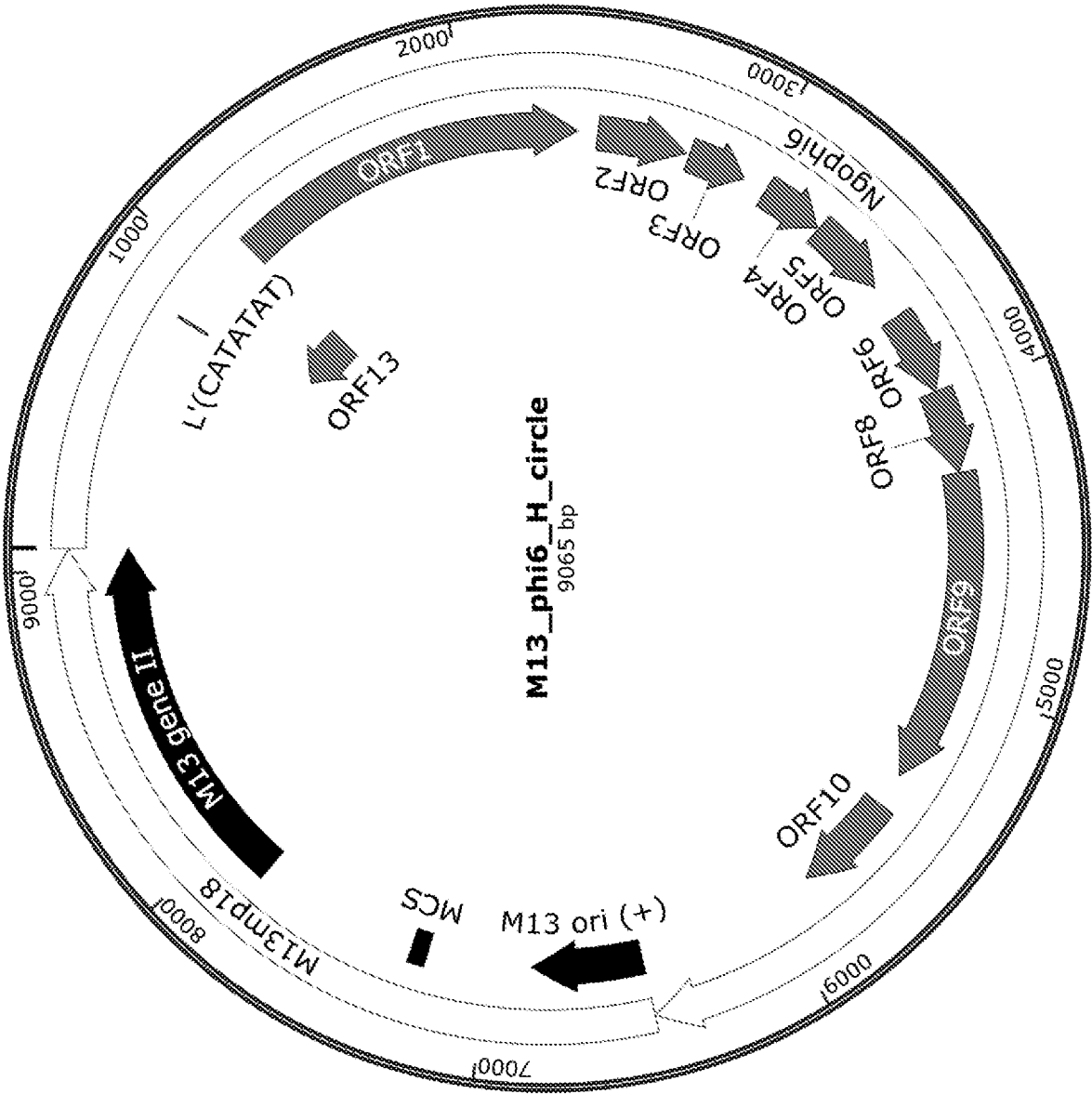


Fig. 15

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Class	Gene type	gene	Example of Required Spacers 5' to 3'	PAM	Penams	Amoxicillin										Carbapenem	Monobactam
						Cephems porin	Cephems n_sporin	Cephems sporin_i	Cephems sporin_ii	Cephems sporin_iii	Cefazidime						
											Cefazolin	Cepharmycin	Cefazidime	Ertapenem	Aztreonam		
A	bl2be _shv2	SHV -a	CCGCGTAGGCATGATAGAAA	TGG	R	R	R								R		
A	bl2be _ctxm	CTXM -b	ACGTTAAACACCGCCATTCC	KGG	R			R	R					R	R		
A	bl2be _ctxm	CTXM -c	GCGCTGGAGAAAAGCAGCGG	RGG	R			R	R					R	R		
A	bl2be _ctxm	CTXM -d	AAGCTGATTGCCCATCTGGG	TGG	R			R	R					R	R		
A	bl2be _ctxm	CTXM -e	ACGCTCAACACCGCGATCCC	CGG	R			R	R					R	R		
A	bl2_tem	TEM-f	AACTACTTACTCTAGCTTCC	CGG	R										R		
A	bl2_kpc	KPC-g	TTGTTGCTGAAGGAGTTGGG	CGG	R		R	R	R					R			
A	bl2a_okp	OKP-h	AGCGAAAAACACCTTGCCGA	CGG	R												
A	bl2_len	LEN-i	CTGGGAAACGGCAGTGAATG	AGG	R												
A	bls_ges	GES-j	TGGGTTGTTGGAGAGAAAAC	TGG	R		R	R	R								
B	bl3_vim	VIM-a	AAACACAGCGGCACCTTCTCG	CGG	R		R	R	R					R			
B	bl3_imp	IMP-b	AAAATTGAAGTTTTTTATCC	MGG	R		R	R	R					R			
C	bl1_ampC		TGGCAGCCGCGAGTGGAGGCC	KGG		R	R	R	R					R			
C	bl1_acc	ACC-1	TCACAGCTACTTGAAGGTTTC	CGG	R		R	R	R					R			
C	bl1_cmy 2	CMY-a	ATCAAAACTGGCAGCCGCAA	TGG		R	R	R	R					R			
C	bl1_cmy 2	LAT-b	ATCAAACTGGCAGCCGCAA	TGG		R	R	R	R					R			
C	bl1_mox	CMY-c	CAGTACTCCAACCCAGCAT	AGG		R	R	R	R					R			
C	bl1_fox	FOX-d	TCACCTGGCCGCAAAATAGTC	TGG		R	R	R	R					R			
D	bl2d _oxa1	OXA-a	ACAACGGATTAAACAGAAGCA	TGG	R												
D	bl2d _oxa2	OXA-2	AGAACATCAGCGCTTGGTCA	AGG	R												
D	bl2d _oxa9	OXA-9	ATAACGGCTTGACCCAGTCA	TGG	R												
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Fig. 16

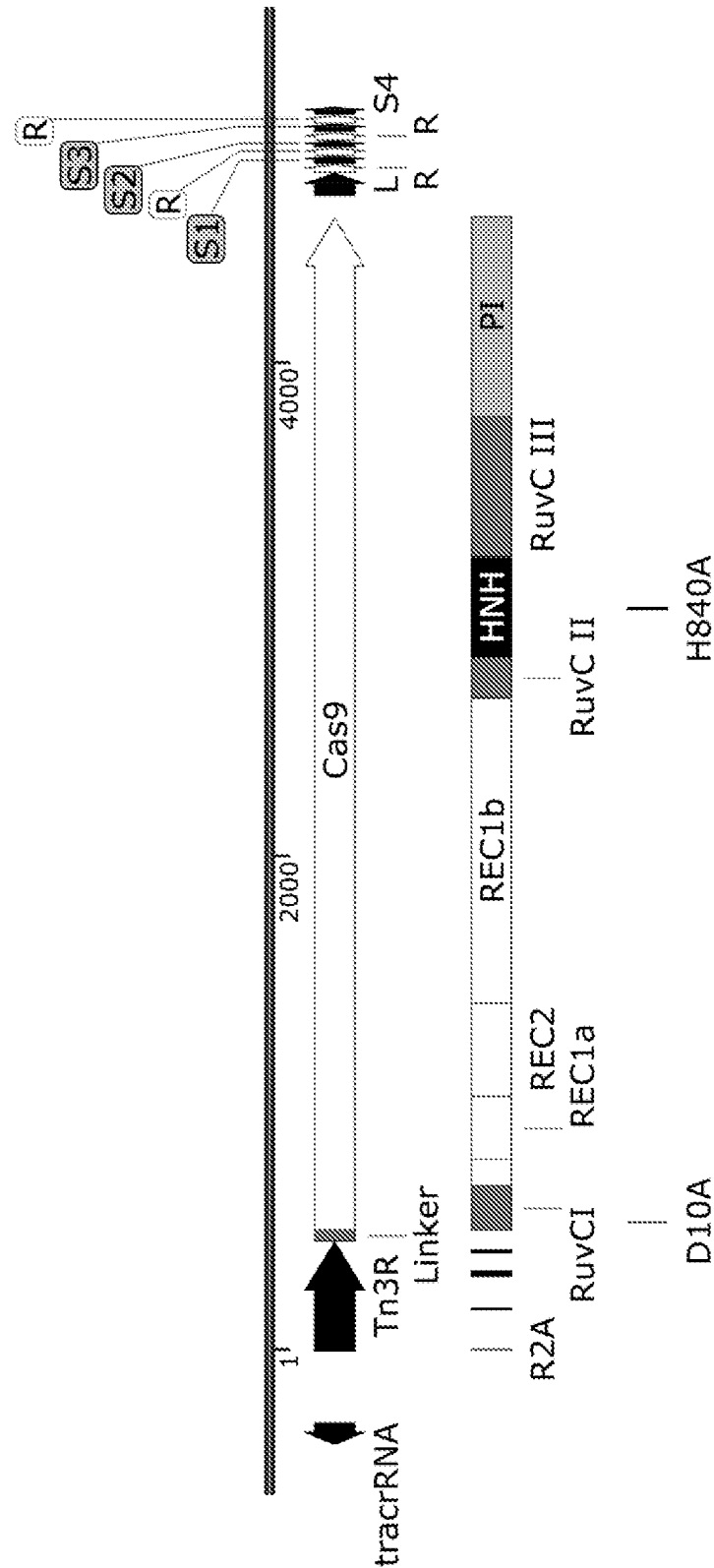


Fig. 18

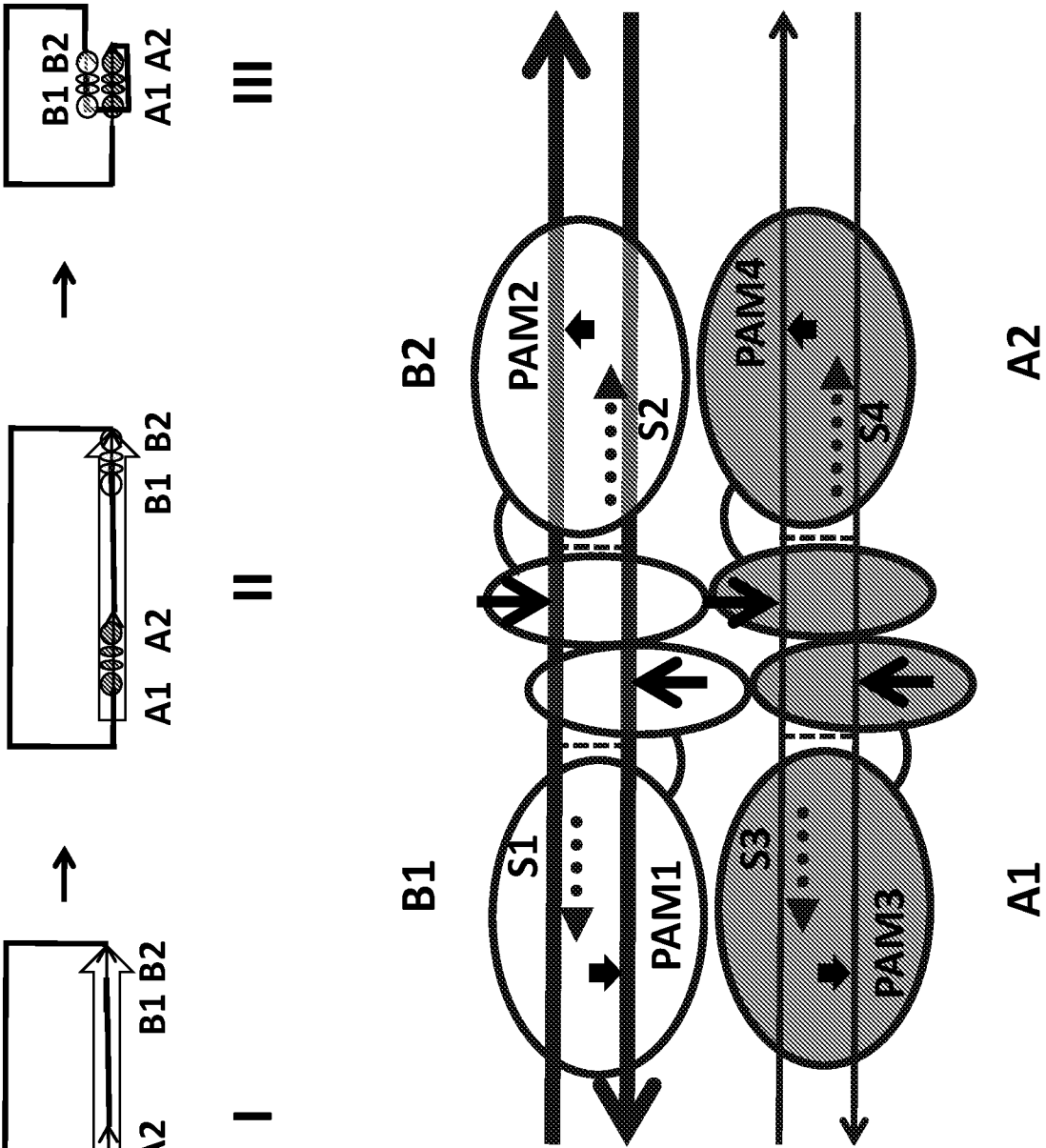


Fig. 19

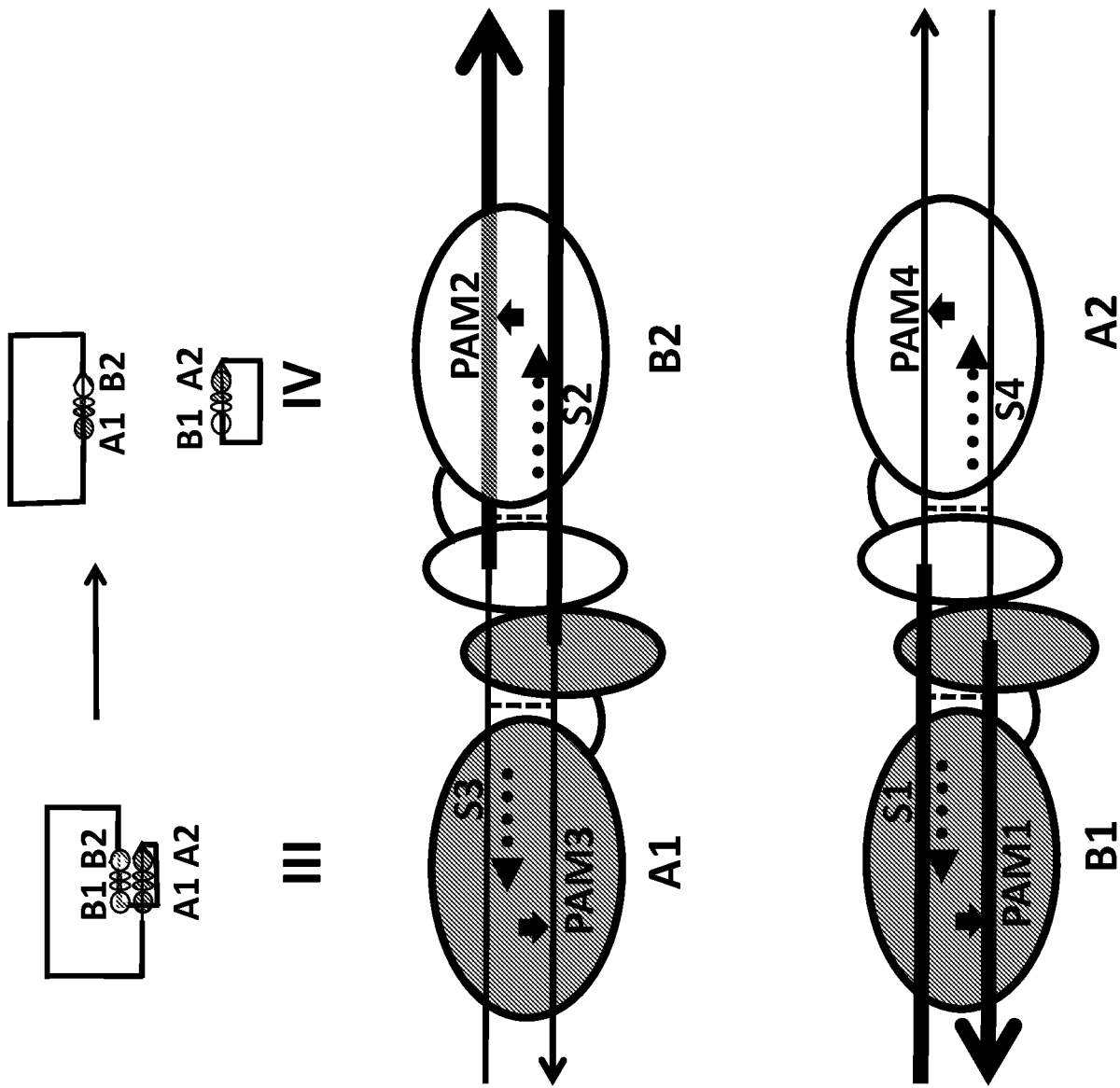


Fig. 20

Class	Gene type	gene	Example of Required Spacer	PAM	Penams	Cephems				Cephams		Carbapenem	Monobactam
						e_Cephalo sporin	n-Cephalo sporin	Cephalo sporin_i	Cephalo sporin_ii	Cephems	Ceftazidime		
5' to 3'													
Amoxicillin													
A	bl2be	SHV	CTGGGAAACGGGAAC	AGG	R	R	R						
A	bl2be	CTXM	ACGTTAAACACCGC	GGG	R		R	R	R		R		R
A	bl2	TEM	AACTACTTACTCTA	CGG	R			R	R				
A	bl2	KPC	TTGTTGCTGAAGGA	CGG	R	R	R	R	R		R	R	
B	bl3	VIM	AAACACAGCGGCAC	CGG	R	R	R	R	R		R	R	
B	bl3	IMP	GGCTAGTTAAAAAT	AGG	R	R	R	R	R		R	R	
B	bl3	NDM	GGTTTGATCGTCAG	CGG	R	R	R	R	R		R	R	
D	bl2	OXA	ATAACGGCCTTGACC	TGG	R								

Fig. 21

Primer	Sequence 5' to 3'
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Fig. 22

pNB104A

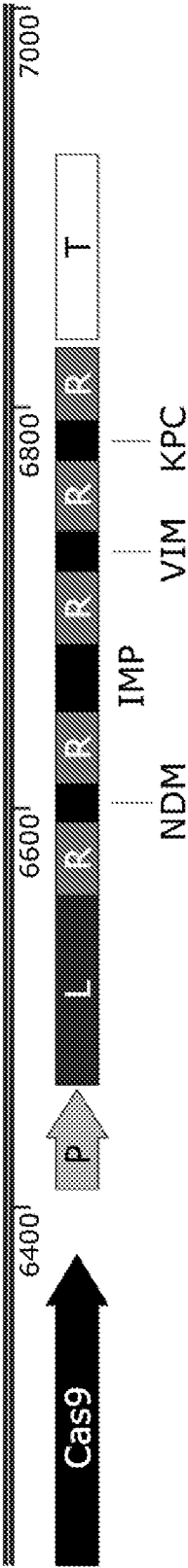


Fig. 23

pNB104B

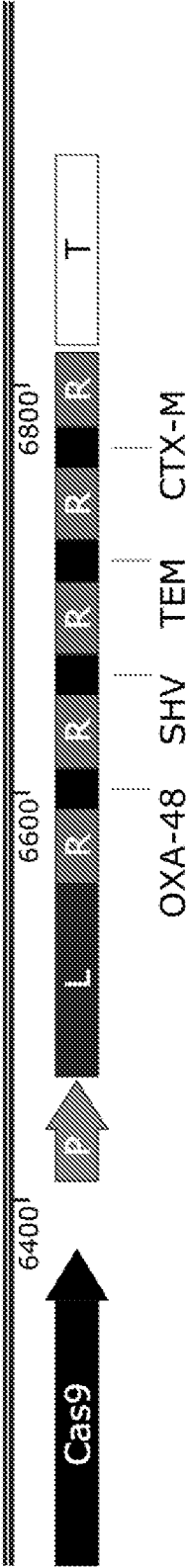


Fig. 24

pNB108



Fig. 25

pNB200

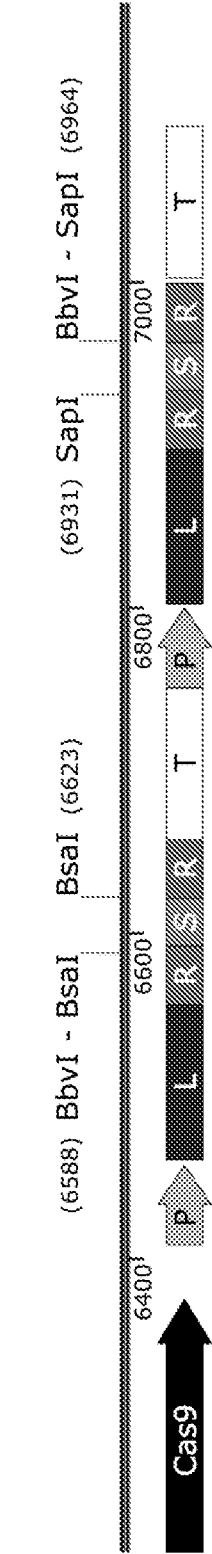


Fig. 26

pNB202

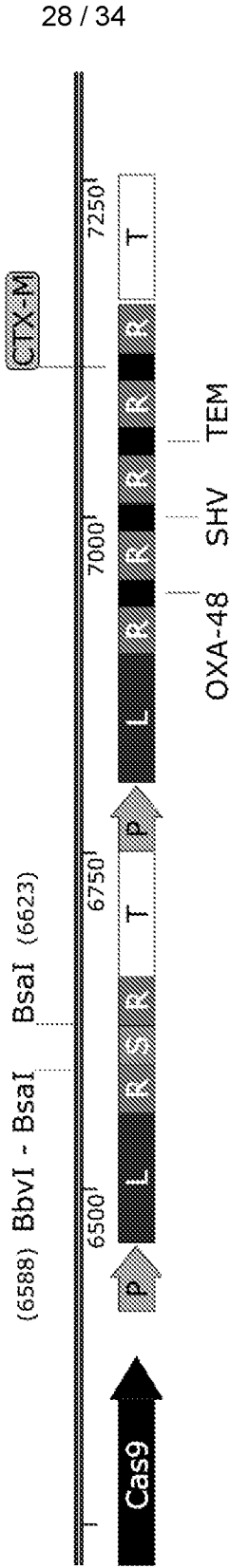


Fig. 27

pNB203

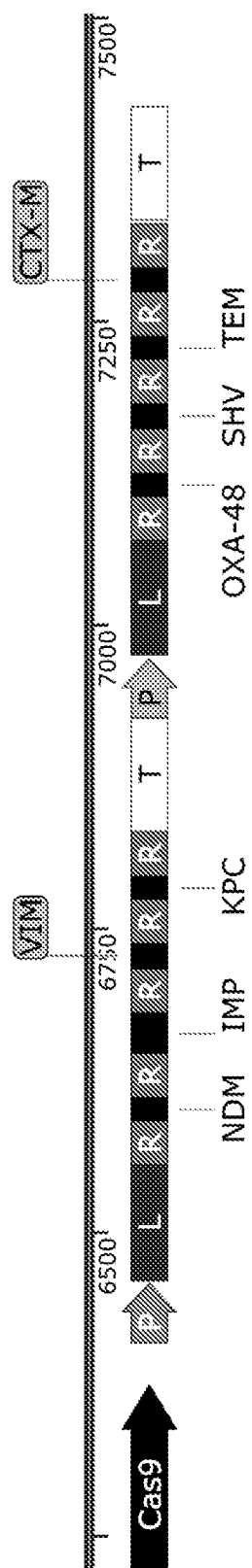


Fig. 28

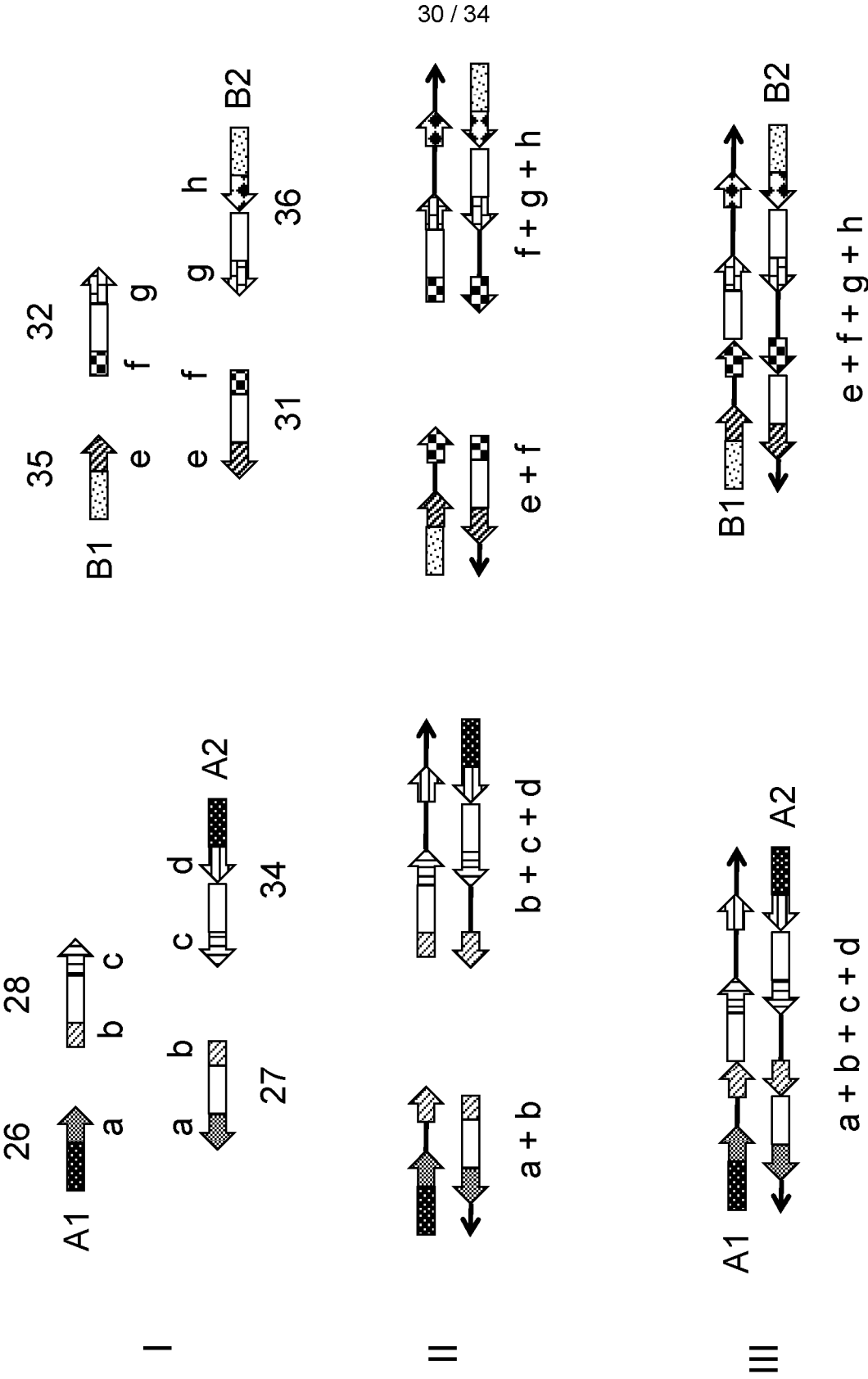


Fig. 29A

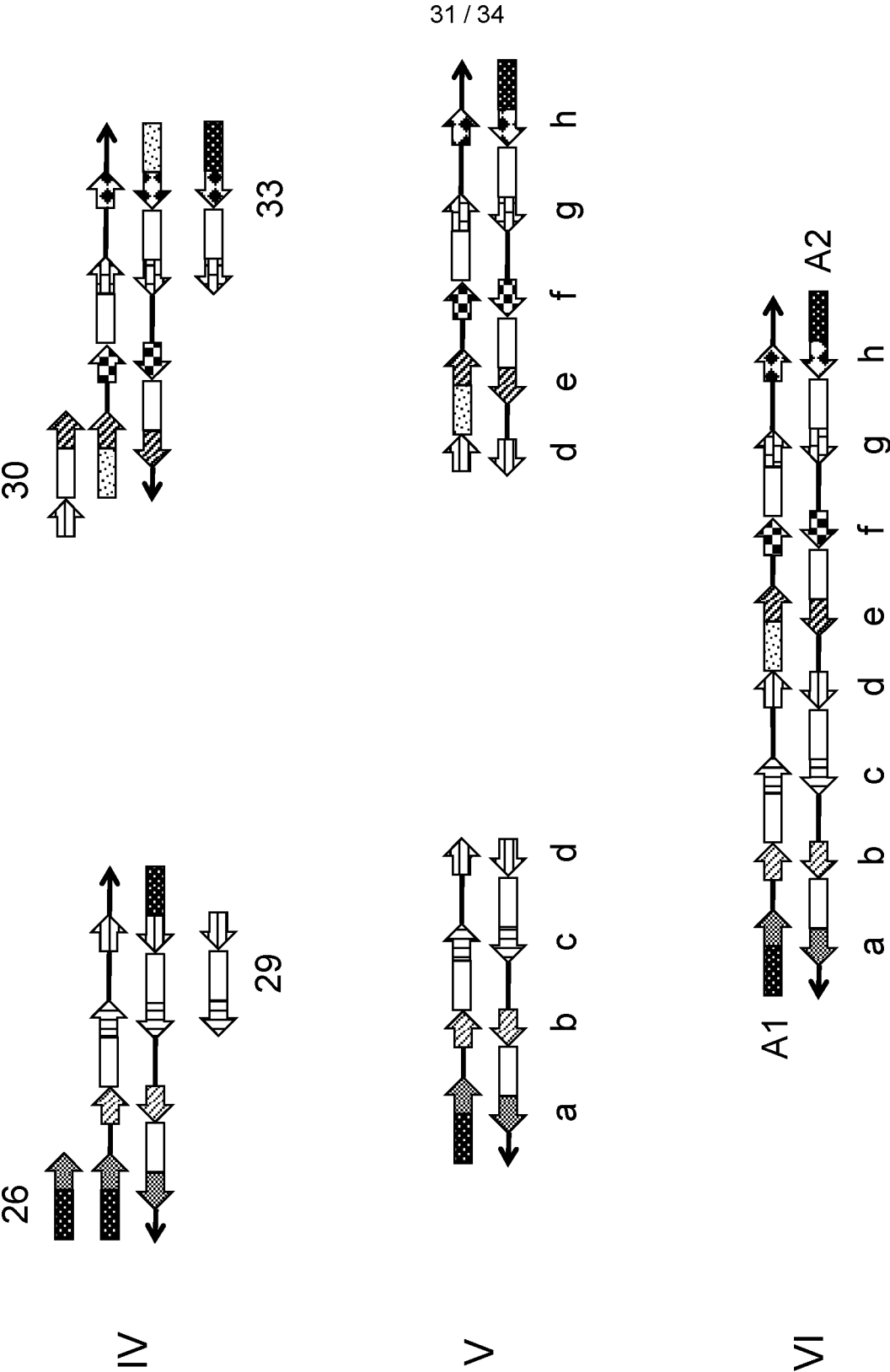


Fig. 29B

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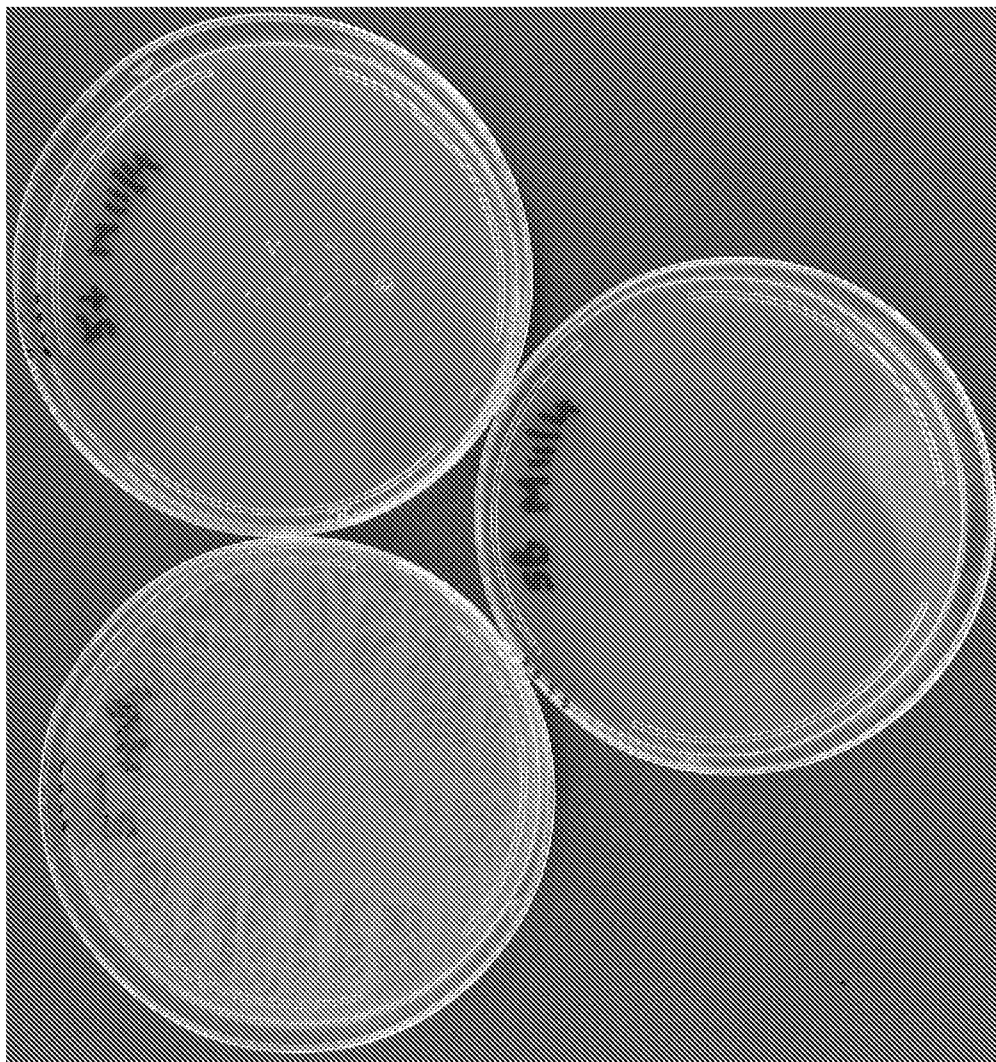


Fig. 30A

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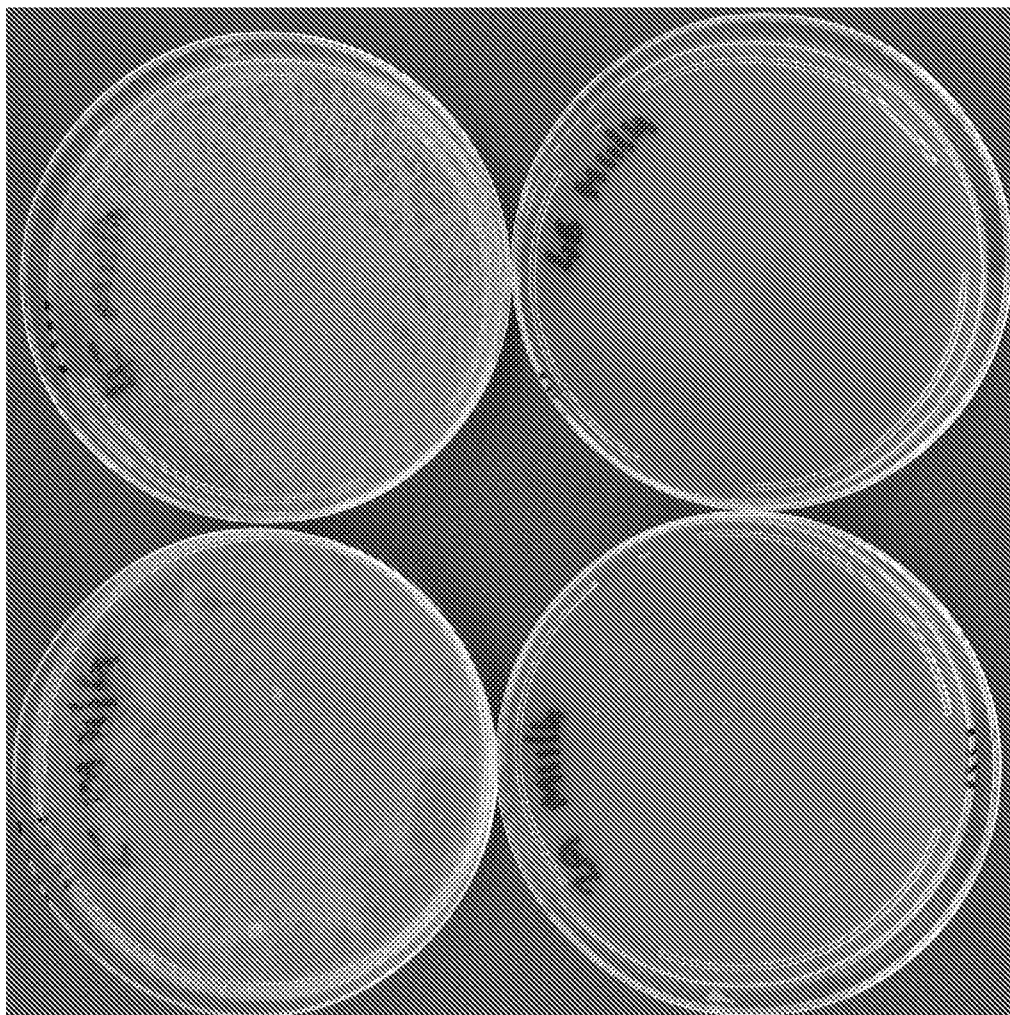


Fig. 30B

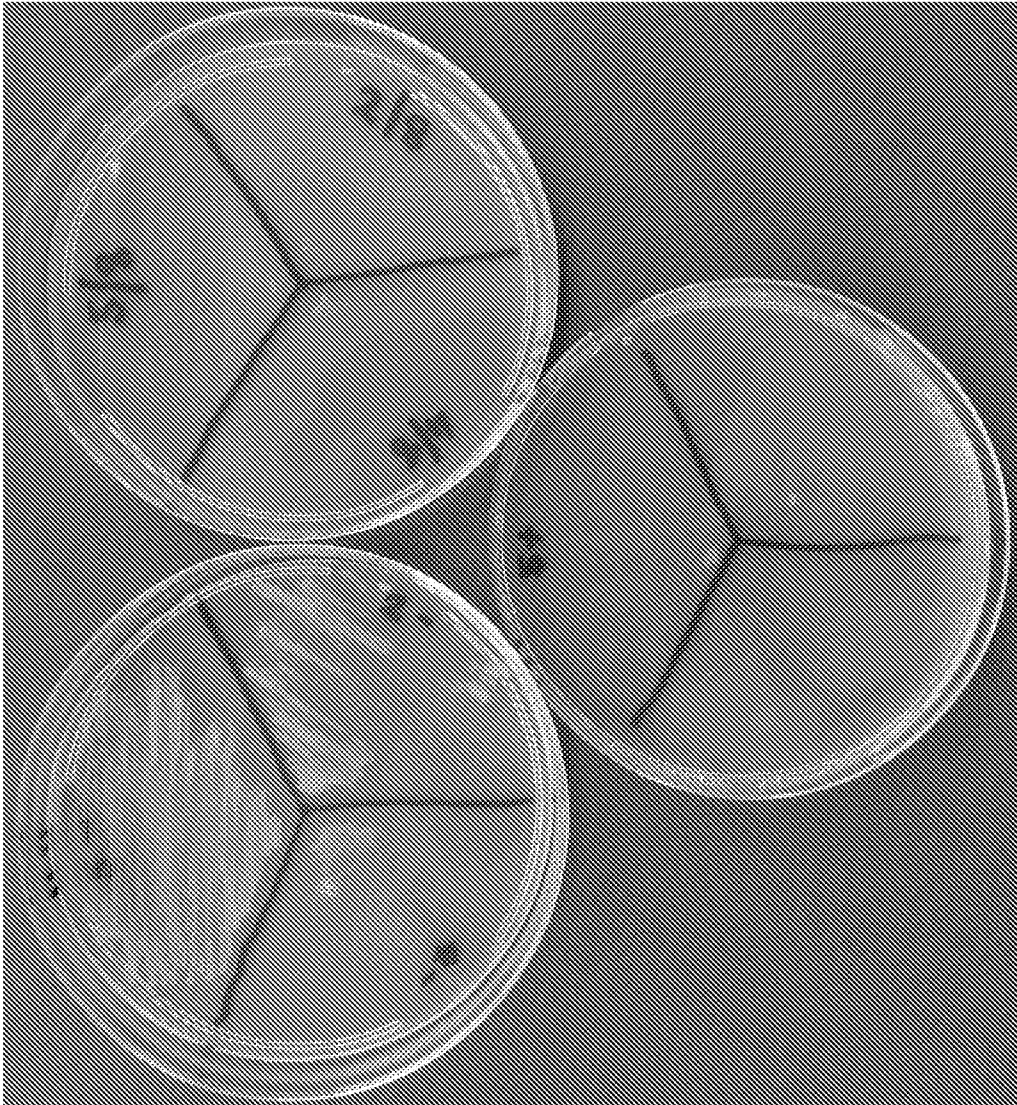


Fig. 30C

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/051132

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/09
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Huang: "E. curi: A way of deactivating antibiotic resistance genes in E. coli", 1 April 2012 (2012-04-01), XP002742963, Retrieved from the Internet: URL:http://uscusj.weebly.com/uploads/4/9/5/ /1/4951238/jonathan_usj_v3.pdf [retrieved on 2015-08-03] the whole document	1-26
X	WO 2010/075424 A2 (UNIV CALIFORNIA [US]; KUNIN VICTOR [US]; YILMAZ SUSAN [US]; SOREK ROTE) 1 July 2010 (2010-07-01) the whole document ----- -/-	1-26



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

10 August 2015

Date of mailing of the international search report

31/08/2015

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No

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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010075424	A2	01-07-2010	NONE
